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(54) Title: TARGETED CYTOTOXIC CELLS

(57) Abstract

Disclosed are mammalian cells that express and secrete immunotoxins directed against tumors, HIV antigens and other diseased cells. Preferred cells include lymphocytes and neurons.

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DESCRIPTION

TARGETED CYTOTOXIC CELLS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the fields of microbiology and immunotherapy. More particularly, it concerns antigen-specific cytotoxic cells which provide antibody-directed and cell-mediated immunity.

2. Description of Related Art

Antibody- and cell-mediated immunities use different mechanisms to defend a host against tumor growth and viral infections. Antibody immunity particularly functions in the neutralization of pathogens through antibody-dependent cell killing (Stites *et al.*, 1994). T-cell mediated immunity utilizes the functions of two T-cell subsets: helper T cells that mediate their effect by the secretion of lymphokines to activate other effector cells, and cytotoxic T cells which kill target cells by the receptor-mediated triggering of apoptosis (Berke, 1994; Young and Liu, 1988).

Increasing numbers of antigens preferentially expressed on the cell surface have been identified for tumor cells, such as oncoproteins and cell differentiation antigens, and these may provide potential markers for the selective destruction of targeted cells in cancer therapy (Urban and Schreiber, 1992; Pastan and FitzGerald, 1991; Boon *et al.*, 1994). Two fundamental approaches for tumor immunotherapy have been explored: the antibody-directed targeting of toxic and cytolytic activity to tumor cells (Vitetta *et al.*, 1987; Waldmann, 1991; Dohlsten *et al.*, 1994), and the augmentation of cellular immune responses to tumors (Perez *et al.*, 1985; Gross *et al.*, 1989; Goverman *et al.*, 1990; Rosenberg, 1991; Eshhar *et al.*, 1993; Hwu *et al.*, 1993). Although antibody-directed immunotherapy has been shown to effectively destroy tumors *in vitro* as well as *in vivo*, a major obstacle to its successful use in cancer therapy is the limited accessibility of antibodies or antibody conjugates to solid tumors (Jain, 1989; Shockley *et al.*, 1992; Reithmuller and Johnson, 1992). In these cases, because of limited access to cancer cells, antibody-directed-immunotherapy cannot completely destroy the targeted tumor. For this type of

immunotherapy to succeed, a successful means of delivering the antibodies or the antibody-conjugates into the interior of the tumor must be found.

Considerable efforts have also been made to stimulate or modify T-lymphocytes by transduction of cytokine genes, or antibody/T-cell receptors to increase the cellular anti-tumor activities (Rosenberg *et al.*, 1988; Rosenberg *et al.*, 1990; Kawakami *et al.*, 1994). But a major limitation in the use of this type of adoptive cellular immunotherapy is the difficulty in obtaining specific cytotoxic lymphocytes (Rosenberg *et al.*, 1988; Rosenberg *et al.*, 1990; Kawakami *et al.*, 1994). Unless specific cytotoxic lymphocytes can be obtained, nonspecific cytotoxicity can occur and result in unacceptable side effects to the patient.

The use of naturally occurring toxins to treat cancers and viral infections currently has a very limited scope. Plants and bacteria produce defensive toxins which are among the most potent toxins capable of killing mammalian cells; *i.e.*, one or a few molecules are sufficient to kill a mammalian cell (Yamaizumi *et al.*, 1978; Chen *et al.*, 1995). For example, plants produce ricin, abrin, gelonin and saporin. Bacterial toxins include *Pseudomonas* exotoxin A (PEA) and diptheria toxin. Toxin molecules, such as PEA, kill a cell by blocking protein synthesis *via* inactivation of the elongation factor-2 (EF-2) in the cytosol (Pastan and FitzGerald, 1992). Unfortunately, the therapeutic use of these and other plant or bacterial toxins is very limited due to the fact that the cytotoxic effects of the toxins are not limited to specific types of cells, such as tumor cells or virally infected cells, and consequently nonspecific cell and tissue death may result in unacceptable side effects or risk to the patient. In addition, these toxins must currently be injected systemically and are cleared from the blood stream and body quite rapidly. For many therapeutic uses, it would be desirable for toxins to remain in the system for an extended period of time so that they might "scavenge" target cells that were not removed or killed by other treatments, for example in the treatment of metastatic tumor cells.

It is evident from the vast research efforts underway to combat cancer and viral diseases that new methods of treating these deadly diseases are needed. In particular, a great benefit would be realized from a method of treatment that improves cytotoxic specificity towards targeted cells, especially cells to which access is limited, such as cells inside solid tumors, and

that allows toxins to remain present in the system as "scavengers" and simultaneously reduces deleterious side effects and risks to the patient.

SUMMARY OF THE INVENTION

The present invention seeks to overcome these limitations by providing a surprising improvement in the field of immunotoxin therapy, in that a subject's own cells or other mammalian cells, may be utilized to produce and secrete an immunotoxin, and those cells may then be re-infused into the subject to act as a therapeutic or preventative agent in the treatment of the targeted disease. The invention provides several improvements over conventional immunotoxin therapies, in which the immunotoxin is prepared synthetically and then injected into a subject. For example, in the practice of the present invention, immunotoxins may be produced in cell culture and conveniently purified from the culture media simply by filtration, centrifugation and chromatography, in contrast to conventional means of producing immunotoxins such as chemical crosslinking of toxins to antibody molecules. Another difficulty with conventional immunotoxins is that they are rapidly cleared from the circulation in a subject, requiring multiple administrations, and making it hard to maintain therapeutic levels in the blood without incurring significant side effects. In contrast, in the practice of the present invention, the immunotoxin producing cells are viable in the body of a subject and produce immunotoxins for periods of weeks to months from a single infusion.

In a certain broad aspect, the present invention may be described as a mammalian cell, such as a cytotoxic-T-lymphocyte, a neuron or other mammalian cell that expresses and secretes an immunotoxin. The immunotoxin is preferably encoded by a vector that comprises a nucleic acid sequence encoding a leader sequence, the expression of which directs the secretion of the immunotoxin out of the cell within a secretory vesicle before the expressed toxin is able to harm the parent, or host cell. Once the immunotoxin is secreted, the cytotoxic effect is primarily limited to those cells expressing the antigen that is recognized by the antibody component of the immunotoxin, and the parent cell, which normally would not express a high level of the antigen, remains viable and continues to express immunotoxin.

A cytotoxic-T-lymphocyte (killer cell) is known in the art as an immune system cell involved in the cellular immune response which has the ability to recognize a foreign antigen and to kill the cell presenting that antigen. An immunotoxin is known in the art as a fusion or conjugation of an antibody component and a toxin component so that the antibody component or domain is able to recognize and bind specifically to an antigen presented on a target cell, for example, and thus direct the toxin to that particular cell type. As used herein, "expresses and secretes" indicates that the cytotoxic-T-cell or other mammalian cell of the invention contains a segment of genetic material encoding an immunotoxin, and that the genetic material is translated into a polypeptide immunotoxin product by the translational mechanisms (i.e. ribosomes) of the host cell, and further that the immunotoxin is transported out of the host cell and secreted into the extracellular media. In the practice of the present invention, the genetic material encoding the immunotoxin may comprise a DNA vector or an RNA vector, or the material may be incorporated into the genomic material of the host cell.

The immunotoxin of the present invention is understood to comprise an antibody component and a toxin component, exemplified herein as co-expressed from a single promoter. However, an immunotoxin of the invention may also be expressed as two or more mRNA messages expressed from separate promoters, whose translation products are then assembled in the cell, or during or after the secretion process. The antibody domain of the immunotoxin may comprise an anti-tumor antibody domain in certain embodiments, or it may comprise an anti-viral antibody domain in certain embodiments or it may comprise an anti-T-helper cell antibody domain of a specific subset of T-helper cells in still other embodiments of the invention. For example, the immunotoxin may immunoreact with a HER2 antigen, a Lym-1 antigen, a growth factor or hormone receptor, or any other antigen known to be overexpressed in a tumor cell. Anti-viral immunotoxins may immunoreact with an HIV antigen such as a gp120 antigen or an influenza virus, a herpesvirus, an Epstein-Barr virus or any known viral antigen. In an alternative embodiment anti-T helper cell immunotoxins may immunoreact with a specific subset of T-helper cells which are involved in an autoimmune disease. Preferred toxins to be used in the practice of the invention include, but are not limited to, *Pseudomonas* exotoxins, such as *Pseudomonas* exotoxin A, ricin A, diptheria toxin, or a portion thereof with cytotoxic and

transport activities, abrin, gelonin, saporin or any other toxin for which the encoding gene is available.

In a certain broad aspect, the invention may comprise a mammalian cell such as a cytotoxic-T-lymphocyte or neuron that expresses and secretes an immunotoxin, where the cell is dispersed in a pharmaceutically acceptable carrier solution. This solution may be suitable for injection, either intramuscularly or intravenously, or it may be suitable for inhalation or other means of administration known in the art. The phrase "pharmaceutically acceptable" refers to compositions that do not produce an allergic or similar untoward reaction when administered to an animal or a human, and would include, but would not be limited to any and all solvents, media, isotonic agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

In another broad aspect, the invention may be described as an animal cell, a lymphocyte, a cytotoxic lymphocyte or a neuron transfected with a vector, such that the vector comprises a nucleic acid sequence operatively linked to a promoter, and the nucleic acid sequence encodes one or more ribosomal binding sites and a fusion protein comprising a leader sequence and an immunotoxin, and all the necessary genetic signals for the expression of a eukaryotic gene such as a polyadenylation site. In this embodiment of the invention, the immunotoxin expressed from the vector is translocated into the endoplasmic reticulum of the lymphocyte as directed by the leader sequence. It is understood therefore, that the leader sequence is expressed prior to the expression of the toxin component of the immunotoxin, and is preferable the first coding region expressed from the promoter. The vector may be any suitable expression vector known in the art, into which the leader and immunotoxin genes are inserted, and may be a plasmid, a viral vector such as an adenoviral, retroviral, or adeno-associated viral vector, a cosmid or other type of vector known in the art. In addition, the vector may comprise a naked DNA or RNA molecule that is associated with a liposome or lipid complex, or the vector may be introduced into a cell by receptor mediated gene transfer, by electroporation or by other mechanical, electrical or chemical means known in the art. The immunotoxin expressed from the vector described above may

immunoreact with a tumor associated antigen, such as HER2 or Lym-1 antigens or with a viral associated antigen, such as an HIV antigen, or even an HIV gp120 antigen or with an antigen associated with a certain subset of T-helper cells. In certain embodiments, the host animal cell, or lymphocyte may comprise a pharmacological composition, when the cell is dispersed in a pharmaceutically acceptable carrier, for example, as described above.

The present invention may be described in a broad aspect as a method of killing a tumor cell, the method comprising contacting the tumor cell with an immunotoxin secreted from an animal cell, such as a cytotoxic-T-cell or a neuron, wherein the antibody portion of the immunotoxin recognizes an antigen of the tumor cell. Preferably the tumor cell overexpresses HER-2 or Lym-1, or other tumor associated antigen and the tumor cell may be a B cell lymphoma, breast, ovarian, gastric or brain tumor cell, or other tumor cell known to overexpress a surface antigen compared to a normal cell. In certain preferred embodiments the tumor cell is in an animal subject, and more preferably in a human subject, or even a human cancer patient, and the animal cell is administered to the subject in a pharmaceutical composition. The invention may also be described in a broad aspect as a method of inhibiting tumor cell, or metastatic tumor cell growth in a subject comprising administering a cytotoxic-T-cell expressing an anti-tumor cell immunotoxin to a subject.

The immunotoxins described herein may also be expressed and secreted by a neuron or other brain cell. The fusion proteins of this embodiment may be described as comprising a peptide signal sequence, an antibody domain and a cytotoxin component, wherein the antibody domain is immunoreactive with an antigen associated with a brain tumor cell, and where the production and secretion of an immunotoxin by the brain cell does not adversely affect the host brain cell as described above. This embodiment is contemplated to be particularly effective in that autologous immunotoxin expressing neurons may be directly implanted into a brain after surgical removal of a tumor, for example, for long-term expression of an immunotoxin against residual tumor cells or metastases.

In a broad aspect, the present invention may also be described as a method of killing a virally infected cell comprising contacting the cell with an immunotoxin expressed from a

cytotoxic-T-cell, or other mammalian cell, wherein the antibody portion of the immunotoxin recognizes an antigen expressed by the virally infected cell. In the practice of this embodiment, a cytotoxic-T-cell may be administered in a pharmaceutical solution to an animal subject or to a human subject having a virally infected cell. In certain broad aspects, the present invention may be described as a method of inhibiting an HIV infection in a subject comprising administering a pharmaceutical solution to the subject, wherein the solution comprises a cytotoxic-T-cell that expresses and secretes an anti-HIV immunotoxin.

In a broad aspect the present invention may also be described as a method of killing a specific species of T-helper cells wherein the specific species of T-helper cells are involved in an autoimmune disease, and the antibody portion of the immunotoxin recognizes an antigen specifically expressed by the particular set of T-helper cells. For example, certain subsets of T-helper cells may be overactive in such conditions as rheumatoid arthritis or systemic lupus erythematosis, for example. The invention offers advantages over therapies that suppress the entire immune system, in that only the specific cells involved in the condition may be targeted and the rest of the immune system is left intact. Another added advantage in the case of rheumatoid arthritis, for example, is that the immunotoxin producing cells may be injected directly into the site of the condition, such as into a joint. In the practice of this embodiment, the cytotoxic lymphocytes, or immunotoxin producing cells may be administered in a pharmaceutical composition to an animal subject, most preferably a human patient who has an autoimmune condition in which a specific subset of T-helper cells is involved.

The present invention may also be described in a broad aspect as a method of producing a recombinant immunotoxin, in which the method comprises the steps of obtaining a mammalian cell transfected with an expression vector encoding a leader sequence and an immunotoxin. Upon introduction of the expression vector into the mammalian cell, or upon transfection of the cell with the expression vector, an immunotoxin is expressed and secreted from the cell into the culture medium. In the practice of this method, the leader-immunotoxin construct may be expressed from a variety of promoters known in the art, including the CMV promoter or even an inducible promoter where desired. The leader may direct the expressed immunotoxin into the endoplasmic reticulum or secretory vesicle of the cell, when the cell is cultured under conditions

effective to express the immunotoxin. The method of producing an immunotoxin may further comprise the step of isolating the immunotoxin from the culture medium. Such a method offers various advantages over chemical synthesis of immunotoxins, or even expression in a bacterial cell such as E. coli. For example, large amounts of imunotoxins may be produced in a continuous cell culture and easily isolated from the culture medium by a variety of methods known in the art. In addition, expression of a mammalian gene in a bacterium often results in insoluble inclusion bodies in which the proteins are not correctly folded and may be inactive. The present invention offers the advantage that active immunotoxins may be easily isolated from the culture medium by standard techniques. These isolated immunotoxins may be packaged for commercial sell, and may be used for *in vitro* applications, or research, or for infusion therapies in which whole cells are not desirable.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1. Schematic representation of construction of anti-HER2/toxin expression vector. The expression vector, pCMV-sFv23e-PE40, contains an anti-HER2 sFv23e gene (Batra *et al.*, 1992; Kasprzyk *et al.*, 1992; Bird *et al.*, 1988; Marasco *et al.*, 1993) with a leader signal sequence, fused to the PE40 sequence (domains II and III) of PEA (ATCC), all under the control of the CMV promoter. In the recombinant retroviral shuttle vector, LNCX-sFve23/PE40, the sFve23/PE40 gene is driven by the internal CMV promoter and the neomycin-resistance (neo) gene is driven by LTR.

FIG. 2. ADP-ribosylation activity of secreted sFv23e-PE40 fusion toxins. The culture media of MOLT-sFv23e-PE40 and MOLT-control were harvested and subjected to ADP-ribosylation assays as described previously (Collier and Kandel, 1971; Chen *et al.*, 1995). The purified PEA proteins were denatured by urea as previously described (Collier and Kandel, 1971) and then used in ADP-ribosylation assays. The mean scintillation counts of the samples

presented are calculated from duplicate determinations after subtracting the background level. The filled columns represent PEA activity at the indicated concentrations. Open columns indicate: culture medium of Molt-4 control; culture medium of MOLT-sFv23e-PE40 (sFv23e-PE40-1); culture medium of MOLT-sFv23e-PE40 concentrated by an Amicon filter (sFv23e-PE40-2).

FIG. 3A. Tumor cells (SKOV-3, N-87) overexpressing HER2, and control cell lines (MCF-7 and NIH3T3) expressing low or undetectable levels of HER2 were seeded onto 96-well plates (1×10^5/ml). After 24 hr incubation, 0.3 ml of Molt-sFv23e-PE40 (clones 1 and 2) or Molt-control cells (5×10^5/ml) was added into each well of the plate, and co-cultures continued for 62 hr. The dead cells in these co-cultures were then scored by trypan blue staining (Chen *et al.*, 1995), and the percentages of selective cell killing are shown after subtracting the percentage of dead cells (3%) in co-cultures of tumor cells with Molt-control cells.

FIG. 3B. Per cent inhibition of cytotoxicity when indicated concentrations of the parental 23e antibody were added to co-cultures of Molt-sFv23e-PE40 (clone 1) and the SKOV3 tumor cells.

FIG. 4A. Tumor cells (N87) (5×10^6) were injected subcutaneously into the flanks of athymic/Nu mice and grown for 16 days. Molt-sFv23e-PE40 or Molt-control cells were washed with PBS and resuspended in culture medium after cell numbers were counted. Mice with tumor xenografts were randomized to two groups: the treatment group (7 mice) was administered Molt-sFv23e-PE40 (1×10^7 in 0.2 ml), and the control group (5 mice) was administered Molt-control cells (1×10^7 in 0.2 ml) on day 1, followed by weekly injections for six weeks. Tumor diameters were caliper-measured every three to five days, and tumor volume was calculated by the formula: Tumor vol = (width)2 × length/2 (Osborne *et al.*, 1985). The mean tumor volumes of the treatment group (filled diamonds connected by solid line) and control group (solid diamonds connected by dotted line) are shown. The differences of growth curves between the treatment and control group were statistically significant (P=0.009), as assessed by a Manova test.

FIG. 4B. Survival of the treatment (solid diamonds) and control (solid squares) groups as described in FIG. 4A are shown.

FIG. 5. Schematic diagram of the anti-gp120/toxin expression vector. To generate an HIV-1-specific killer cell, a neutralizing human monoclonal antibody (f105) which recognizes the CD4-binding site of HIV-1 gp120 expressed on the surface of HIV-1 infected cells was used. The gene (PE40) which encodes domain II (for translocation across membrane bilayer) and domain III (for adenosine diphosphate (ADP)-ribosylation of EF-2) of PEA was fused to the κ chain gene of F105 . The resultant bicistronic vector (pCMV-Fab105-PE40) contains an Fd chain ($V_H + C_{HI}$), internal ribosomal entry site (IRES) and the κ-PE40 chimeric gene preceded by a leader signal sequence under the control of the cytomegalovirus (CMV) promoter. In the LNCX-Fab105-PE40 recombinant retroviral shuttle vector, the Fab105/PE40 chimeric gene is expressed from the internal CMV promoter, and the Neo gene is expressed from LTR. The construct was identified by restriction enzyme digestion and confirmed by DNA sequence analysis.

FIG. 6A. Antigen binding activity of the secreted Fab105-PE40 fusion proteins detected by ELISA. Positive binding activities to HIV-1 gp120 were detected in the medium of Jurkat-Fab105-PE40, while no significant binding activity was observed in the medium of Jurkat-control. Solid columns are: culture medium of Jurkat-Fab105-PE40 (Fab105-PE40); culture medium of Jurkat-control (Jurkat). Open columns: Positive binding activities to HIV-1 gp120 by indicated concentrations of purified F105 antibody (0.8 to 0.1μg/ml).

FIG. 6B. ADP-ribosylation activity (DPM) of secreted Fab105-PE40. The culture medium of Jurkat-Fab105 and Jurkat-control cells were subjected to ADP-ribosylation assays. Solid columns are: culture medium of Jurkat-Fab105-PE40 (Fab105-PE40-1); culture medium of Jurkat-Fab105-PE40 concentrated by Amicon filtration (Fab105-PE40-2); culture medium of Jurkat-control (Jurkat). Open columns: ADP-ribosylation activity of secreted Fab105-PE40 measured by indicated concentrations of purified PEA (5 to 40 ng per reaction).

FIG. 7A. Selective cytotoxicity to HIV-1 infected cells *in vitro* by transduced lymphocytes. Parental Jurkat cells were infected with a laboratory strain HIV-1 virus (IIIB) and two primary patient isolates (INME and TPO), and the reverse transcriptase (RT) activities were measured every three to four days until RT activity reached a plateau. The HIV-1-infected Jurkat cells were then placed in the top-chambers of 12-well Costar-Transwell filter tissue culture plates, and Jurkat-Fab105-PE40 or Jurkat-control cells were placed in the bottom chambers at ratios of 1:1(HIV-infected cells: Jurkat-Fab105-PE40 or Jurkat-control cells) (open columns), and 1:10 (solid columns). Viable cell numbers of HIV-1-infected cells in the top chambers were counted at 72 hr post-co-culture, and the percentages of cell killing are expressed as percent of viable cell numbers of the infected cells co-cultivated with Jurkat-Fab105-PE40 as compared with viable cell numbers of the infected cells co-cultivated with Jurkat-control.

FIG. 7B. Selective cell killing of transduced LAK cells. Human LAK cells were generated by incubating peripheral blood lymphocytes in a culture medium supplemented with riL-2 and PHA for 48 hours. The LAK cells were transduced with the Fab105-PE40 gene by cocultures with the transfected packaging cell line (PA317) for 24 hours, followed by expansion for several days. The transduced LAK cells were cocultivated with HIV-infected cells from a laboratory strain of HIV-1 (IIIB) and two primary patient strains (INME and TPO) for 72 hours, and the percentages of cell killing are presented as described above. Open column: 1:1 ratio of HIV-infected cells: transduced or mock-transduced LAK cells. Solid column: 1:10 ratio of HIV-infected cells: transduced or mock-transduced LAK cells.

FIG. 7C. Inhibition kinetics of HIV-1 infection. Parental Jurkat cells infected with the patient primary HIV-1 isolate (WEAU) were co-cultivated with Jurkat-Fab105-PE40 or Jurkat-control cells at ratios of 1:1, 1:5, and 1:10. RT activities in the co-cultures were monitored every three to four days post-cocultivation.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The present invention provides a new class of cytotoxic cells which combine the specificity of antibodies, extreme potency of toxins, and the effector cell-properties of lymphocytes, such as homing and tissue penetration, thus offering the advantages of both

antibody-directed and cell-mediated immunotherapy. The cytotoxic lymphocytes not only recognize the target, but also produce potent molecules to kill the target cells in a major histocompatibility (MHC)-independent manner. In addition, the described cells are not adversely affected by the immunotoxins produced. Surprisingly, the cytotoxic lymphocytes not only remain viable but do not appear to be impaired in any way.

The present invention may also be described as cytotoxic cells which are able to express and secrete an immunotoxin which comprises a leader signal sequence, an immunologically active antibody domain, which recognizes an antigen specific to the target cell, and a cytotoxin. The immunotoxins produced by the cells of the described invention have high specificity towards their target cells and tissues such that no nonspecific toxic interaction with nontarget cells or tissues is detected. The cells are able to produce the immunotoxin molecules for a sustained period of time, up to three months or more.

A further aspect of the immunotoxin secreting cells of the present invention is that the cells may be designed with a negative selection marker for reasons of patient safety. By incorporating a marker gene into the recombinant vector that is used to transduce the cells, such as two tandem copies of the herpes simplex virus thymidine kinase gene (HSV-tk) that provide negative selection in the presence of ganciclovir (Ishibashi *et al.*, 1993), the transduced cells may be selectively destroyed if necessary. This would allow the clinician to selectively destroy the immunotoxin secreting cells in the event of adverse side effects.

This new class of cytotoxic cells, which are able to produce and secrete novel targeted toxin proteins, was designed based on the knowledge of protein trafficking and the cell killing mechanism of toxins. Toxin molecules, such as *Pseudomonas* exotoxin A (PEA), block cellular protein synthesis and thus kill the cell by inactivating the elongation factor-2 (EF-2) in the cytosol (Vitetta *et al.*, 1987; Waldmann, 1991; Dohlsten *et al.*, 1994; Yamaizumi *et al.*, 1978; Hwang *et al.*, 1987; Siegall *et al.*, 1989; Pastan *et al.*, 1992), indicating that toxin molecules have to be located in the cytosol in order to kill a cell. The present invention exploits this property, by genetically modifying a cell to produce and secrete targeted toxins using a leader signal sequence (Walter and Lingappa, 1986). Subsequently, newly synthesized, targeted toxins are translocated

into the lumen of the endoplasmic reticulum (ER) cotranslationally and then secreted out of the cells. The toxin-expressing cells should remain viable since the interaction of the synthesized fusion toxins with the EF-2 in the cytosol is blocked by the membrane lipid bilayer of the ER and secretory vesicles. The secreted targeted toxins selectively bind and destroy target cells after being internalized and released into the cytosol (Vitetta *et al.*, 1987; Waldmann, 1991; Dohlsten *et al.*, 1994; Yamaizumi *et al.*, 1978; Hwang *et al.*, 1987; Siegall *et al.*, 1989; Pastan *et al.*, 1992). However, the secreted toxins are unable to kill the toxin-expressing cells because of lack of target antigens on the cell surface. The genetically modified cells have potent and selective cytotoxicity to the targeted cells indicating that this approach may have broad application for the treatment of viral infection, cancers and autoimmune diseases.

This approach of the present invention may be used in therapeutic applications for treating a viral infection, in particular HIV-1 infection, wherein the transduced lymphocytes may home back and secrete anti-HIV/toxins in lymphoid tissues and organs, the major reservoirs of HIV-1 infection (Pantaleo *et al.*, 1993; Embretson *et al.*, 1993). Thus, relatively high levels of targeted toxins produced locally may be more effective in neutralizing cell-free virions and destroying HIV-infected cells, compared to systemic administration of antibody/toxin proteins (Chaudhary *et al.*, 1988). The approach of the present invention can also be used to selectively destroy other targets, such as tumors, since increasing numbers of antigens selectively expressed on the surface of tumor cells have been identified by antibodies (Pastan and FitzGerald, 1992; Walter and Lingappa, 1986; Waldmann, 1991). For example, breast cancer cells can be recognized and destroyed by transduced cytotoxic cells secreting targeted toxins.

A certain embodiment of the present invention may be useful in the treatment of certain autoimmune diseases and reactions, such as rheumatoid arthritis. This type of disease is often aggravated by a specific subset of overactive T-helper cells. The present invention provides a method of specifically inhibiting the deleterious T-cells by directing an immunotoxin to only those cells. This provides an advantage over therapies for autoimmune diseases in which the entire immune system is inhibited, because the targeted immunotoxin will not affect other elements of the subject's immune response mechanisms, including other subsets of T helper cells that do not cross-react with the antibody. In the practice of this embodiment, the deleterious

species of T-helper cells may be isolated from a subject, and used to produce an antibody which recognizes a specific antigen to the subset of T-helper cells by standard techniques of antibody production known in the art. This antibody would then be used as described herein to produce an immunotoxin vector and the vector would then be used to transduce a lymphocyte, for example that would secrete the immunotoxin targeted to the T helper cells. The transduced cells may also be infused directly into the site of inflammation, such as a joint, in order to be even better targeted to the site of the disease. In summary, this new class of antigen-specific cytotoxic cells and fusion proteins with the features of antibody-directed and cell-mediated immunotherapy may have wide applications for treatment of viral infection, cancer and autoimmune diseases and reactions.

As shown herein, the transduced cytotoxic cells were found to have potent and selective cytotoxicity to targeted cancer cells *in vitro* as well as *in vivo*. In addition to combining the specificity of antibodies to the potency of toxin molecules and the effector-cell-properties of lymphocytes, these cells are shown to have specific anti-tumor activity *in vivo* without apparent non-specific toxicity. An aspect of the invention is the use of tumor infiltrating lymphocytes (TILs) or other lymphocytes, since TILs can proliferate rapidly *in vitro* and recirculate in and localize at tumor sites after reinfusion (Perez *et al.*, 1985; Gross *et al.*, 1989; Goverman *et al.*, 1990; Rosenberg, 1991; Eshhar *et al.*, 1993; Hwu *et al.*, 1993; Rosenberg *et al.*, 1988; Rosenberg *et al.*, 1990; Kawakami *et al.*, 1994; Bolhuis *et al.*, 1991; Topalian *et al.*, 1989; Barth *et al.*, 1990). The tumor-homing characteristic of transduced TILs may allow them to function not only as a vehicle for delivering antibody/toxins to tumor tissues, but also as a producer of targeted toxins within the tumor tissues. It is especially appealing that tumor micrometastases which are almost impossible to detect can be selectively destroyed by these targeted cytotoxic cells when reinfused back into patients, acting as an immune surveillance.

Immunotoxins

Immunotoxin technology is fairly well-advanced and known to those of skill in the art of antibody research. Immunotoxins are agents which have an antibody component linked to another agent, particularly a cytotoxic or otherwise anticellular agent, having the ability to kill or suppress the growth or cell division of cells. Exemplary anticellular agents include

chemotherapeutic agents and radioisotopes as well as cytotoxins. Examples of chemotherapeutic agents are hormones such as steroids; antimetabolites such as cytosine arabinoside, fluorouracil, methotrexate or aminopterin; anthracycline; mitomycin C; vinca alkaloids; demecolcine; etoposide; mithramycin; or antitumor alkylating agents such as chlorambucil or melphalan.

Preferred immunotoxins often include a plant-, fungal- or bacterial-derived toxin, such as an A chain toxin, a ribosome inactivating protein, α-sarcin, aspergillin, restrictocin, a ribonuclease such as placental ribonuclease, angiogenin, diphtheria toxin or *Pseudomonas* exotoxin, saporin, gelonin, or abrin to mention just a few examples. It is an important element of the present invention that the toxin be co-expressed genetically with the antibody in the lymphocyte or other mammalian cell. Therefore, preferred toxins to be used are those for which the encoding genes are known and which can be inserted into an expression vector by standard techniques of molecular biology. The use of any such toxin in the practice disclosed herein would be encompassed by the scope and spirit of the claimed invention.

For example, a biologically active ricin A chain has now been cloned and expressed (O'Hare *et al.*, 1987; Lamb *et al.*, 1985; Halling *et al.*, 1985), so that ricin A, or smaller or otherwise variant peptides which nevertheless exhibit an appropriate toxin activity may be used in the practice of the present invention. Moreover, the fact that ricin A chain has now been cloned allows the application of site-directed mutagenesis, through which one can readily prepare and screen for A chain derived peptides and obtain additional useful moieties for use in connection with the present invention.

Monoclonal Antibody Generation

Means for preparing and characterizing antibodies are well known in the art (See, e.g., *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic composition, such as a specific species

of T-helper cells, in accordance with the present invention (either with or without prior immunotolerizing, depending on the antigen composition and protocol being employed) and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobenzoyl-N-hydroxysuccinimide ester, carbodiimide and bis-diazotized benzidine.

As is also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis)*, incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, may also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate MAbs.

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable animal with a selected immunogen composition, e.g., a purified or partially purified T-helper cell surface protein, polypeptide or peptide or any other desired antigenic composition. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are preferred animals, however, the use of rabbit or sheep cells is also possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the potential for producing antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generating protocol. These cells may be obtained from biopsied spleens, tonsils or lymph nodes, or from a peripheral blood sample. Spleen cells and peripheral blood cells are preferred, the former because they are a rich source of antibody-producing cells that are in the dividing plasmablast stage, and the latter because peripheral blood is easily accessible. Often, a panel of animals will have been immunized and the spleen of animal with the highest antibody titer will be removed and the spleen lymphocytes obtained by homogenizing the spleen with a syringe. Typically, a spleen from an immunized mouse contains approximately 5×10^7 to 2×10^8 lymphocytes.

The antibody-producing B lymphocytes from the immunized animal are then fused with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures preferably are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas).

Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984). cites). For example, where the

immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and 4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter *et al.* (1977). The use of electrically induced fusion methods is also appropriate (Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8}. However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key

enzymes of the salvage pathway, e.g., hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MAbs. The cell lines may be exploited for MAb production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. The individual cell lines could also be cultured *in vitro*, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. MAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

Immunoassays

Immunoassays to be used in the practice of the present invention include, but are not limited to those described in U.S. Patent No. 4,367,110 (double monoclonal antibody sandwich assay) and U.S. Patent No. 4,452,901 (western blot). Other assays include immunoprecipitation of labeled ligands and immunocytochemistry, both *in vitro* and *in vivo*.

Immunoassays, in their most simple and direct sense, are binding assays. Certain preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and other solid support immunoassays known in the art. Most preferred are ELISAs as described by Doellgast *et al.* (1993, 1994) and by U. S. Patent No. 4,668,621. Immunohistochemical detection using tissue sections and radioimmunoassays (RIA) are also particularly useful. However, it will be readily appreciated that detection is not limited to such techniques, and Western blotting, dot blotting, FACS analyses, and the like may also be used.

In one exemplary ELISA, the antibodies of the invention, i.e., those to be used in the production of an immunotoxin, are immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a biological sample suspected of containing the target antigen(s), which may itself be linked to a detectable label, is added to the wells. After binding and washing to remove non-specifically bound immunecomplexes, the amount of bound antigen(s) may be determined.

Alternatively, the first added component that becomes bound within the primary immune complexes may be detected by means of a second binding ligand that has binding affinity for the primary antibody. In these cases, the second binding ligand may be linked to a detectable label. The second binding ligand is itself often an antibody, which may thus be termed a "secondary" antibody. The primary immune complexes are contacted with the labeled, secondary binding ligand, or antibody, under conditions effective and for a period of time sufficient to allow the formation of secondary immune complexes. The secondary immune complexes are then generally washed to remove any non-specifically bound, labeled secondary antibodies or ligands, and the remaining label in the secondary immune complexes is then detected. This type of ELISA is a simple "sandwich ELISA".

Further methods include the detection of primary immune complexes by a two step approach. A second binding ligand, such as an antibody, that has binding affinity for the primary antibody is used to form secondary immune complexes, as described above. After washing, the secondary immune complexes are contacted with a ~~third binding ligand or antibody~~ that has binding affinity for the second antibody, again under conditions effective and for a period of time

sufficient to allow the formation of immune complexes (tertiary immune complexes). The third ligand or antibody is linked to a detectable label, allowing detection of the tertiary immune complexes thus formed. This system may provide for signal amplification if desired.

In another exemplary ELISA, the samples suspected of containing the target antigen(s) are immobilized onto the well surface and then contacted with antibodies or immunotoxins of the invention. After binding and washing to remove non-specifically bound immunecomplexes, the bound antigen(s) are detected. Where the initial antibodies are linked to a detectable label, the immunecomplexes may be detected directly. Again, the immunecomplexes may be detected using a second antibody that has binding affinity for the first immunotoxin antibody(ies), with the second antibody being linked to a detectable label.

Another ELISA in which the proteins or peptides are immobilized, involves the use of antibody competition in the detection. In this ELISA, labeled antibodies are added to the wells, allowed to bind, and detected by means of their label. The amount of target antigen(s) in an unknown sample is then determined by mixing the sample with the labeled antibodies before or during incubation with coated wells. The presence of the target antigen(s) in the sample acts to reduce the amount of immunotoxin antibody(ies) available for binding to the well and thus reduces the ultimate signal.

Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immunecomplexes. These are described as follows:

In coating a plate with either antigen or antibody, one will generally incubate the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a nonspecific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein and solutions of milk powder. The coating allows for blocking of nonspecific

adsorption sites on the immobilizing surface and thus reduces the background caused by nonspecific binding of antisera onto the surface.

In ELISAs, it is probably more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of a protein or antibody to the well, coating with non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the control antigen and/or biological sample to be tested under conditions in a manner conducive to allow immunecomplex (antigen/antibody) formation. Detection of the immunecomplex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

"Under conditions in a manner conducive to allow immunecomplex (antigen/antibody) formation" may include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of nonspecific background. The "suitable" conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are typically from about 1 to 2 to 4 hours, at temperatures preferably on the order of 25° to 27° C, or may be overnight at about 4° C or so.

Following all incubation steps in an ELISA, the contacted surface is washed so as to remove non-complexed material. A preferred washing procedure includes washing with a solution such as PBS/Tween, or borate buffer. Following the formation of specific immunecomplexes between the test sample and the originally bound material, and subsequent washing, the occurrence of even minute amounts of immunecomplexes may be determined.

To provide a detecting means, the second or third antibody will have an associated label to allow detection. Preferably, this will be an enzyme that will generate color development upon incubating with an appropriate chromogenic substrate. Thus, for example, one will desire to contact and incubate the first or second immunecomplex with a urease, glucose oxidase, alkaline phosphatase or hydrogen peroxidase-conjugated antibody for a period of time and under

conditions that favor the development of further immunecomplex formation (e.g., incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

After incubation with the labeled antibody, and subsequent to washing to remove unbound material, the amount of label is quantified, e.g., by incubation with a chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid [ABTS] and H_2O_2, in the case of peroxidase as the enzyme label. Quantification is then achieved by measuring the degree of color generation, e.g., using a visible spectra spectrophotometer.

Promoters and Enhancers

The promoters and enhancers that control the transcription of protein encoding genes in mammalian cells are composed of multiple genetic elements. The cellular machinery is able to gather and integrate the regulatory information conveyed by each element, allowing different genes to evolve distinct, often complex patterns of transcriptional regulation.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator proteins. At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV 40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start

site as well. The spacing between elements is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins. The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities. They have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

Below are a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the immunotoxin construct. Additionally any promoter/enhancer combination (AS PER the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the immunotoxin fusion gene in a Gene Therapy protocol.

Table 1

ENHANCER	REFERENCES
Immunoglobulin Heavy Chain	Hanerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1988; Kiledjian *et al.*, 1988; Porton *et al.*, 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria *et al.*, 1987, Winoto and Baltimore, 1989; Redondo *et al.*, 1990
HLA DQ a and DQ ß	Sullivan and Peterlin, 1987
ß-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1985
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5	Koch *et al.*, 1989
MHC Class II HLA-DRa	Sherman *et al.*, 1989
ß-Actin	Kawamoto *et al.*, 1988; Ng *et al.*, 1989
Muscle Creatine Kinase	Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989a
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase *I*	Omitz *et al.*, 1987
Metallothionein	Karin *et al.*, 1987; Culotta and Hamer, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987

Table 1 (Continued)

ENHANCER	REFERENCES
Albumin Gene	Pinkert *et al.*, 1987, Tronche *et al.*, 1989, 1990
a-Fetoprotein	Godbout *et al.*, 1988; Campere and Tilghman, 1989
t-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
ß-Globin	Trudel and Constantini, 1987
e-fos	Cohen *et al.*, 1987
c-HA-ras	Triesman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsch *et al.*, 1990
a_1-Antitrypain	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990

Table 1 (Continued)

ENHANCER	REFERENCES
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987 Schaffner *et al.*, 1988
Polyoma	Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; deVilliers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a,b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Chol *et al.*, 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987, Stephens and Hentschel, 1987; Glue *et al.*, 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe *et al.*, 1988; Rowen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp and Marciniak, 1989; Braddock *et al.*, 1989

Table 1 (Continued)

ENHANCER	REFERENCES
Cytomegalovirus	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

Table 2

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger and Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987; Karin ®, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors and Varmus, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Fonta *et al.*, 1985; Sakai *et al.*, 1986
ß-Interferon	poly(rI)X poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angle *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angle *et al.*, 1987b

Table 2 (Continued)

Element	Inducer	References
SV40	Phorbol Ester (TFA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez *et al.*, 1988
a-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2kb	Interferon	Blanar *et al.*, 1989
HSP70	Ela, SV40 Large T Antigen	Taylor *et al.*, 1989; Taylor and Kingston, 1990a,b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone a Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

Use of promoters and enhancers

It is understood in the art that to bring a coding sequence under the control of a promoter, one positions the 5' end of the transcription initiation site of the transcriptional reading frame of the protein between about 1 and about 50 nucleotides "downstream" of (i.e., 3' of) the chosen promoter. In addition, where eukaryotic expression is contemplated, one will also typically desire to incorporate into the transcriptional unit which includes the cotransporter protein, an appropriate polyadenylation site (e.g., 5'-AATAAA-3') if one was not contained within the original cloned segment. Typically, the poly A addition site is placed about 30 to 2000 nucleotides "downstream" of the termination site of the protein at a position prior to transcription termination.

Recombinant Vectors

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from cytomegalovirus (CMV), polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers *et al.*, 1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the Hind III site toward the Bg1 I site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

The origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from CMV, SV40 or other viral (*e.g.*, Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Construction of Expression Vectors

The DNA fragment encoding amino acids 253 to 613 of PEA (ATCC) was fused in frame to the κ gene of the anti-HIV-1 gp120 human monoclonal antibody F105. The bicistronic expression vector containing the Fd-IRES-κ-F105 was constructed previously (Chen *et al.*, 1994), and used to construct the expression vector pCMV-Fab105-PE40 (FIG. 5). The resultant bicistronic expression vector, pCMV-Fab105-PE40, contains the FdF105 gene/internal ribosome entry site (IRES) sequence/k105-PE40 fusion gene under the control of a CMV promoter. This expression vector was identified with restriction enzyme digests, and confirmed by DNA sequence analysis.

In this expression vector, a single mRNA is transcribed under the control of the CMV promoter, and two gene products are translated independently from a single mRNA. The translation of the first Fd105 gene is cap-dependent, and the second κ-PE40 fusion gene is

translated under the control of the IRES in a cap-independent manner. A more detailed description of the vector construction follows.

Construction of bicistronic expression vector for Fab105 using IRES

Co-expression of the heavy and light chains of the Fab105 fragments was achieved from a Fab105 expression vector using two independent CMV promoters (Chen *et al.*, 1994). The co-expression vector of the Fab105 expression cassette with two identical CMV promoter sequences was further modified to include an internal ribosome entry site (IRES) sequence. IRES derived from EMCV has been used for efficient co-expression of two or more genes in retroviral and other vectors. In this kind of expression vector, a single mRNA is transcribed under the control of an upstream promoter, and two gene products are translated independently from a single mRNA. The translation of the first gene is cap-dependent, and the second one is translated under the control of IRES in a cap-independent manner.

The bicistronic Fab105 expression vector using an internal ribosome entry site (IRES) sequence was constructed from the expression vector using two identical CMV promoter as follows: The IRES sequence from the encephalomyocarditis virus (EMCV) was PCR™-amplified from a pCITE-2a plasmid (Novogen, Madison, WI) using the primers (5'-TTTGCTAGCGGTATTATCATCGTG-3' (SEQ ID NO:1) with an additional *NheI* cloning site; 5'-TTTGCGGCCGCGAATTAATTCCGGTTA-3' (SEQ ID NO:2) with an additional *NotI* site). The IRES DNA fragment, about 515 bp, was purified from an agarose gel, and then cut with *NheI/NotI.*

To introduce a unique *NheI* cloning site into the bicistronic expression vector with IRES, a light (lambda) chain fragment of a human neutralizing monoclonal antibody 2.1H against the CD4-binding site of HIV-1 gp120 was PCR™-amplified from the cDNA of the hybridoma and sequenced (Bagley *et al.*, Molecular Immunology, 31:1149-1160, 1994). The 2.1H light-chain fragment was then cut with *NheI/XbaI* and gel-purified. The *NheI/XbaI* cut-2.1H light chain and *NotI/NheI* cut-IRES DNA fragments were cloned into the *NotI/XbaI*-cut pCMV-Fab105 by a three-piece ligation. The resultant construct, pCMV-F105-IRES-2.1H, which contains the Fd of F105, IRES, and 2.1H light chain sequence under the control of the cytomegalovirus (CMV)

promoter, was identified and confirmed by DNA sequencing. The pCMV-Fd105-IRES-2.1H plasmid was then digested with *NheI* and *XbaI*, and the vector DNA fragments were recovered from an agarose gel. The κ chain DNA fragments of F105 were PCR™-amplified from pCMV-Fab105 using the primers 5'-GGTTGCTAGCATGGAAACCCCAGCGCAG-3' (SEQ ID NO:3) 5'-AAAATCTAGATTAACACTCTCCCCTGTTGAA-3' (SEQ ID NO:4). The amplified κ chain fragments, about 760 bp, were digested with *NheI* and *XbaI*, and purified from agarose gels. The *NheI*/*XbaI*-cut pCMV-Fd105-IRES-2.1H, and -κ DNA fragments were then ligated together and transformed into a host *E. coli*. The resultant bicistronic expression vector, pCMV-Fab105-I which contains the Fd/IRES/κ genes under the control of CMV promoter, was identified by enzyme digestions, and used for further study.

Construction of the bicistronic expression vector for Fab105-PE40 fusion protein

The PEA gene obtained from the American Type Culture Collection (ATCC) contains three functional domains: Domain I, cell-recognition; Domain II, translocation domain (amino acid (aa) residues 253 to 404); and Domain III, catalytic domain (aa of 405 to 613) (Pastan and FitzGerald, Science. 254:1173-1177, 1992). The *Bgl*II-*Eco*RI fragments containing the sequence encoding amino acids 253 to 613 of PEA were cut from the plasmid pJH8 (ATCC), and cloned into the pSP72 vector (Stratagene) (pSP-PE40). To incorporate a *NotI* site into the fragment, a primer corresponding to amino acid residues 253 to 258 of PEA with an additional *NotI* site (5'-TTGCGGCCGCGAAAGGCGGCAGCCTGGCCGCG-3' (SEQ ID NO:5) and a reverse primer corresponding to the amino acids 330 to 324 (5'-GCGGATCGCTTCGCCCAGGT-3' (SEQ ID NO:6) were used to amplify the amino acids 253 to 330, and the amplified DNAs were then cut with *NotI*/*Sal* I. The *Sal* I/*XbaI*-DNA fragment containing the sequence aa 308 to 613 of PEA was cut and purified from the pSP-PE40 vector. The *NotI*/*Sal* I fragment of aa 253/308 and *Sal* I/*XbaI* DNA fragment encoding aa 308/613 was then cloned into the *NotI*/*XbaI* sites of pCMV-sFv23e-S by three-piece ligation. The *NotI*/*XbaI* DNA fragment containing the PE40 (domain II and III) sequence was cut from the pCMV-sFv23e/PE40, and the κ chain gene was PCR™-amplified from the pCMV-Fab105-I vector to incorporate *NheI*/*NotI* sites. The resultant bicistronic expression vector for Fab105-PE40 was constructed by three-piece ligation of the *NheI*/*NotI*-cut κ chain/*NotI*/*XbaI*-cut PE40/*NheI*/*XbaI*-cut pCMV-Fab105-I. The vector was identified by restriction enzyme digestion and confirmed by DNA sequencing.

Radiolabelling and Immunoprecipitation

For transient expression, COS cells were transfected with 20 μg of plasmid DNA pCMV-Fab105-PE40 or vector pRc/CMV using Lipofectin (Chen and Compans, 1991) and, radiolabeled with 200 μCi of ^{35}S-cysteine for various times after 60 to 72 hr transfection. Transduced Jurkat cells (5×10^6) were radiolabeled with 200 μCi of ^{35}S-cysteine for various times. The culture medium and cell lysates were immunoprecipitated with a mixture of the anti-PEA and -human IgG. The samples were analyzed by electrophoresis on SDS-polyacrylamide gels under reducing conditions (Chen and Compans, 1991), and then visualized using a Phosphoimager (Molecular Dynamic).

Cell Transduction and PCR™ Amplification

Jurkat cells, $CD4^+$ human T-lymphocytes, were transfected with pCMV-Fab105-PE40 by electroporation, and selected in medium containing G418 (800 μg/ml) for two to three weeks (Chen *et al.*, 1994). Genomic DNA was extracted from the cells as described (Maniatis *et al.*, 1986). The following oligonucleotides were used for the PCR™ reactions: Pair A: 5′-TTATTGCTAGCGTCGACCTTCGCGATGTACGGGCCAG-3′ (SEQ ID No:7) and 5′-GGTACCGAATTCTCTAGAACAAGATTTGGGCTC-3′ (SEQ ID NO:8) Pair B: 5′-GGTAGGCCTCAGGTGCAGCTGCAGGAG-3′ (SEQ ID NO:9) and 5′-TTTGCTAGCGGTATTATCATCGTG-3′ (SEQ ID NO:10) Pair C: 5′-TTTGCGGCCGCGAATTAATTCCGGTTA-3′ (SEQ ID NO:11) and 5′-TTTAAGATCTCCACACTCTCCCCTGTTGAAGCT-3′ (SEQ ID NO:12) Pair D: 5′-TTGAATTCGGAGGTGGCGGAAGTCACCCTGGCGCGGAGTTC-3′ (SEQ ID NO:13) and 5′-TTTATCGATTCTAGATTACGGCGGTTTGCCGGGCTG-3′ (SEQ ID NO:14). The PCR™ products were analyzed on agarose gels.

Enzyme-Linked Immunosorbent Assay and ADP-Ribosylation Assay

ELISA was performed in HIV-1 gp120-coated plates as described elsewhere (Chen *et al.*, 1994, incorporated herein by reference). Briefly, HIV-1 gp120 coated plates were incubated with culture medium from Jurkat-Fab105-PE40 or Jurkat-control, followed by reaction with the anti-human IgG (Sigma).

As shown in FIG. 7A, the secreted Fab105-PE40 has specific binding activity to HIV-1 gp120. To further determine the binding activity, ELISA was performed using other antibodies. An Elisa microtiter plate was coated with 10 μg of recombinant HIV-1 gp120 (American Bio-Technologies Inc.). The plate was blocked with TBST buffer containing 1% BSA. Jurkat-Fab105-PE40 or Jurkat-control culture medium was added to the plate. Goat anti-human Immunoglobulin (Sigma) or anti-PEA (GIBCO BRL) in TBST at 1:1,000 dilution was then added to the wells, followed by an anti-goat IgG-Peroxidase Conjugate (Sigma) in TBST-1% BSA at 1:1,000 dilution. A positive binding activity was detected in the culture medium of Jurkat-Fab105-PE40, either using the anti-human IgG or anti-PEA; while no significant binding activity was found in the Jurkat-control culture medium.

Cell Culture and HIV-1 Infection

To examine the cell-killing activity of the cytotoxic cells, 12-mm-diameter, 0.40-μM-pore-size Costar-Transwell filters, which would separate the cells, but not large molecules, were placed in 12-well plates (Costar Corp., Cambridge, MA) and used for the co-cultivation assay. Parental Jurkat cells were infected with a laboratory strain HIV-1 virus (IIIB) or two primary patient isolates (INME and TPO), which are able to infect Jurkat cells and were obtained from Drs. F. Gao and G. Shaw at UAB (Birmingham, AL). Reverse transcriptase was measured every three to four days until RT reached its plateau. The HIV-1-infected Jurkat cells were then placed in the top-chamber, and Jurkat-Fab105-PE40 or Jurkat-control cells were placed in the bottom chamber at different ratios. Viable cell numbers of HIV-1-infected cells in the top chambers were counted daily. Cell killing activity was expressed as percent of viable infected cells co-cultivated with Jurkat-Fab105-PE40 compared to viable infected cells co-cultivated with Jurkat-control. To examine the effects of the transduced cells on HIV-1 RT production, the primary HIV-1 isolate (WEAU) was used to infect parental Jurkat cells. At day 6 post-infection, a high level of RT activity in the culture medium from the infected Jurkat cell culture was detected. These infected cells were then washed with PBS, resuspended in RPMI-1640 medium/10% bovine serum at a cell density of 5×10^5/ml. The infected Jurkat cells were then mixed with Jurkat-Fab105-PE40 or Jurkat-control at several different ratios. RT assays were performed as described previously (Poiesz *et al.*, 1980).

Construction of Target-Specific Cytotoxic Cells

Tumor-specific cytotoxic cells were generated by transducing an anti-HER2/toxin expression vector into human lymphocytes. The genetically modified cells remained viable and expressed and secreted targeted antibody/toxins, since the interaction of the newly synthesized fusion toxins with the EF-2 in the cytosol was blocked by the membrane lipid bilayer of the ER and secretory vesicles. The secreted antibody-toxin fusion proteins then recognized and destroyed target cells after internalization and release into the cytosol of the targeted tumor cells. However, the targeted toxin proteins did not kill the transduced toxin-expressing cells which lacked the target antigens on the cell surface. Thus, this new class of cytotoxic cells with the features of both antibody-directed and cell-mediated cytotoxicity will have broad applications for cancer and other disease therapy.

The HIV-specific cytotoxic cells were generated by transducing an anti-HIV gp120/toxin expression vector into human lymphocytes. The genetically modified cells expressed and secreted the targeted antibody/toxins, which recognize and specifically kill the targeted HIV-1-infected cells. This antigen-specific cytotoxic cell functions as a targeted toxin-producer as well as a carrier, thus combining the advantages of antibody-directed and cell-mediated immunotherapy.

Protein Synthesis Rates of Target-specific Lymphocytes

To determine whether cellular protein synthesis is blocked in cells expressing the antibody/toxin protein, ^{3}H-leucine incorporation rates of Jurkat-Fab105-PE40, Jurkat-control cells, MOLT-SFv23e-PE40 or MOLT-control were examined. Jurkat-Fab105-PE40 or Jurkat-control(0.5×10^{6}) were washed with RPMI 1640 medium and resuspended in 1 ml of RPMI medium 1640 containing 10% FBS. ^{3}H-leucine (ICN Pharmaceuticals, Inc., 1 mCi/ml) was added to the medium at a final concentration of 4 μCi/ml, incubated for 1 hr at 37°C, followed by three washes with PBS to remove unincorporated ^{3}H-leucine. After precipitation and washing with trichloroacetic acid (TCA), the pellets were resuspended in 4 ml of scintillation liquid (ScintiVerse BD, Fisher), and counted on a scintillation counter (LS Beckman). There were comparable levels of ^{3}H-leucine incorporation in Jurkat-Fab105-PE40 and Jurkat-control cells.

Thymidine Incorporation Rates of Target-Specific Lymphocytes

DNA synthesis rates were determined by measuring thymidine incorporation. Jurkat-F105-PE40, Jurkat-control cells, MOLT-F105-PE40 or MOLT-control cells were washed with RPMI-1640, and resuspended in RPMI-1640/10% FBS at a density of 0.5×10^6ml. The cells were then added with or without PHA (Vector Laboratories) at a final concentration of 0.8 μg/ml for 1 hr. 10 μCi of (methyl-^{3}H)-thymidine (ICN Pharmaceuticals, Inc., 1 mCi/ml) were then added to each of the cell cultures, and incubated at 37°C for 12 hr. The cells were washed three times, and solubilized with 100 μl of 0.5% SDS. The solubilized cell lysates were then added to 4 ml of scintillation liquid, and counted on a scintillation counter. There were no significant differences of ^{3}H-thymidine incorporation in Jurkat-F105-PE40 or Jurkat-control. In addition, both of the cell lines were over 95% viable as determined by trypan blue exclusion. It was also found that the Jurkat-Fab105-PE40 or Jurkat-control cells had a similar proliferation curve. The Jurkat-Fab105-PE40 cells remained viable with normal proliferation during the observed period of over four months.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1

Anti-Tumor Cytotoxic Cells

The following example demonstrates the construction and use of a cytotoxic cell specific for HER-2, a transmembrane protein of the epidermal growth factor family, which is overexpressed in various human tumors, such as breast, ovarian, gastric and other cancers (King *et al.*, 1985; Slamon *et al.*, 1989; Wen *et al.*, 1992; Hynes, 1993; Muss *et al.*, 1994). An anti-

HER2 single-chain antibody (sFv) comprising the heavy chain variable region linked to the light chain variable region derived from a monoclonal antibody 23e was found to have a high-affinity binding activity to the extracellular domain of HER-2, and to be efficiently internalized after binding (Batra *et al.*, 1992; Kasprzyk *et al.*, 1992; Bird *et al.*, 1988; Marasco *et al.*, 1993). In this study, the sFv23e gene with a leader signal sequence was used to fuse in-frame with a truncated gene (PE40) encoding domains II (translocation) and III (catalytic) (amino acids 253 to 613) of PEA (Rosenberg *et al.*, 1988; Rosenberg *et al.*, 1990; Kawakami *et al.*, 1994; Gary *et al.*, 1984; Allured *et al.*, 1986) under the control of a cytomegalovirus (CMV) promoter (FIG. 1). This vector, pCMV-sFv23e-PE40, expresses and secretes anti-HER2sFv-PE40 fusion toxins in mammalian cells.

A forward primer for amplification of the sFv23e gene with an additional signal leader sequence including a *Hind*III cloning site was synthesized: (5′-TTAAGCTTATGAAACATCTGTGGTTCTTCCTTCTCCTGGTGGCAGCTCCCAGATGG GTCCTGTCCGACGTCCAGCTGACC-3′ (F-1) (SEQ ID NO:15) and a reverse primer with an additional *NotI* cloning site (5′-TTTGCGGCCGCGGAGACGGTGACCGTGGT-3′ (SEQ ID NO:16) were used to amplify the sFv23e gene with an additional leader signal sequence. The sFv23e gene with the leader sequence fragments was then cloned into the *Hind*III/*NotI* sites of plasmid pRc/CMV (Invitrogen), and the resultant vector was designated as pCMV-sFv23e-S. The *Bgl*II-*Eco*RI fragment containing the sequence encoding amino acids (aa) 253 to 613 of PEA was cut from the plasmid pJH8 (ATCC), and cloned into the pSP72 vector (Stratagene) (pSP-PE40). To incorporate a *NotI* site into the fragment, a primer corresponding to the 253 to 263 aa with an additional *NotI* site (5′-CCCGCGGCCGCGCCGTCGCCGAGGAACTC-3′ (F-2)(SEQ ID NO:17), and a reverse primer corresponding to amino acids 330 to 322 (5′-GCGGATCGCTTCGCCCAGGT-3′ (SEQ ID NO:18) were used to amplify DNA fragments encoding amino acids 253 to 322, and the amplified DNAs were then cut with *NotI*/*SalI*. The *SalI*/*Xba* DNA fragment encoding amino acids 322 to 613 of PEA was cut and purified from the pSP-PE40 vector. The *NotI*/*SalI* fragment of amino acids 253/322 and *SalI*/*XbaI* fragment of amino acids 322/613 were then cloned into the *NotI*/*XbaI* sites of pCMV-sFv23e-S by three-piece ligation. The resultant construct was identified by restriction enzyme digestion and confirmed by DNA sequence analysis.

To determine whether mammalian cells are able to produce the antibody/toxin fusion proteins, COS cells were transfected with pCMV-sFv23e-PE40 DNA, and analyzed by immunofluorescent staining. Strong positive staining in the cytoplasm, especially in the perinuclear Golgi region, of the transfected cells was observed when cells were incubated with either an anti-sFv23e (Batra *et al.*, 1992; Kasprzyk *et al.*, 1992; Bird *et al.*, 1988; Marasco *et al.*, 1993), or anti-PEA (Gibco-BRL) antibody indicating that transfected mammalian COS cells expressed a high level of the anti-HERSsFv/PE40 fusion proteins. This staining pattern resembles a typical secretory protein pattern (Chen *et al.*, 1994). The fluorescent-positive cells showed normal morphology, and over 98% of the transfected cells were viable, similar to the control cells. No significant staining was observed in the control cells transfected with vector only and stained with a mixture of the anti-sFv23e and -PEA antibodies.

The expression of the fusion protein was further examined by radiolabeling and immunoprecipitation analyses. COS cells were transfected with pCMV-sFv23e-PE40 DNA or a control vector, and 60 hours later, the transfected cells were radiolabeled with ^{35}S-cysteine for 4 hours (Chen and Compans, 1991). The antibody-toxin fusion proteins, about 70 kd, were immunoprecipitated from the culture medium of the transduced cells by either the anti-sFv23e or anti-PEA antibodies followed by SDS-PAGE analysis. No corresponding protein band was found from the control cells. These results confirmed that mammalian cells are able to produce the antibody/toxin fusion proteins.

Cytotoxic cells specific for HER-2 were then generated by transducing pCMV-sFv23e-PE40 into human T-lymphocytes (Molt-4 cells), followed by G418 selection (Chen *et al.*, 1994). Human lymphocytes express undetectable levels of the HER-2 protein (Potter *et al.*, 1989; Press *et al.*, 1990). G418-resistant cells were picked and subcloned, and further analyzed for their biological features and protein expression. Pulse-chase radiolabeling studies showed that the fusion proteins were produced and secreted from the transduced cells.

Genomic PCR™ analyses were used to detect whether the expression vector gene was incorporated. Genomic DNAs were isolated from MOLT-sFv23e-PE40 and MOLT-control cells

(Maniatis *et al.*, 1986), and PCR™ amplification was performed. The sFv23e DNA fragment (about 770 bp), the domain II (about 450 bp) or domain III (about 610 bp) of PEA DNA fragments were specifically amplified from the genomic DNA of transformed MOLT-sFv23e-PE40 cells, but not from the control cells. Furthermore, a significant ADP-ribosylation activity in the culture medium of MOLT-sFv23e-PE40 was detected, but only a background level of ADP-ribosylation activity was found in the medium of control cells, indicating that the secreted fusion proteins have toxin enzymatic activity. When compared to the activity of purified PEA (Gibco-BRL), over 0.5 μg of PEA protein was produced from the MOLT-sFv23e-PE40 at 24 hr/1×10^6 cells/ml.

Several biological features of MOLT-sFv23e-PE40 were examined. First, when cell proliferation rates and viability of MOLT-sFv23e-PE40 and MOLT-control were examined, there was no apparent difference. Second, the DNA synthesis rates of MOLT-sFv23e-PE40 and MOLT-control were similar, as measured by ^{3}H-thymidine incorporation with or without PHA (0.8 μg/ml) stimulation. Third, the protein synthesis rates of MOLT-sFv23e-PE40 and MOLT-control were nearly identical as measured by ^{3}H-leucine incorporation. Thus, the transduced MOLT-sFv23e-PE40 cells maintained their basic biological functions.

To determine whether the transduced cells exhibit selective cytotoxicity to targeted tumor cells, co-cultivation studies were performed. Cell lines expressing different levels of HER2 were co-cultivated with MOLT-sFv23e-PE40 and MOLT-control lymphocytes for 62 hr. Significant cell killing was observed in the tumor cells (SKOV-3 and N-87) overexpressing HER-2 (Batra *et al.*, 1992; Kasprzyk *et al.*, 1992; Bird *et al.*, 1988; Marasco *et al.*, 1993; Kraus *et al.*, 1987) co-cultivated with MOLT-sFv23e-PE40, while only a small percentage of cells were killed in the co-cultures of MOLT-sFv23e-PE40 and the tumor cells (MCF-7 and NIH/3T3) expressing low levels of HER-2 (Di Fiore *et al.*, 1987) (FIG. 3A). MOLT-control cells had no significant effects on the tumor cells either expressing high or low-levels of HER-2. Therefore, the protein synthesis of HER2-overexpressing tumor cells co-cultivated with MOLT-sFv23e-PE40 was selectively inhibited. The parental 23e antibody was also shown to inhibit the cytotoxicity in a concentration dependent manner (FIG. 3B). Collectively, these results indicate that the transduced lymphocytes have selective cytotoxicity to the targeted cancer cells.

The transformed lymphocytes, MOLT-sFv23e-PE40, and control lymphocytes, were generated by transfection of plasmid DNA followed by G418 selection (Chen et al., 1994). Genomic DNAs were extracted from the cell lines as described (Maniatis, 1986). The oligonucleotides used for the PCR™ reactions are listed below: Pair A:; F-1, and 5'-TTTAAGATCTACAGGAGACGGTGACCGTGG-3' (SEQ ID NO:19) Pair B: F-2, and 5'-TTGCGGCCGCGAAAGGCGGCAGCCTGGCCGCG-3' (SEQ ID NO:5) Pair C: 5'-GGTACCGAATTCTCTAGAGGCGACGTCAGCTTCAGC-3' (SEQ ID NO:20), and 5'-TTAATTGCGGCCGCTTACTTCAGGTCCTCGCG-3' (SEQ ID NO:21). The PCR™-amplified DNA fragments were analyzed on agarose gels.

EXAMPLE 2

In vivo Activity of Anti-Tumor Cytotoxic Cells

The anti-tumor activity of the transduced cells described in Example 1 was further determined in a nude mouse model. The human gastric cancer cell line, N87, which has been shown to overexpress HER-2 proteins and grow well as a subcutaneous tumor in nude mice (Batra *et al.*, 1992; Kaspryzk *et al.*, 1992; Bird *et al.*, 1988; Marasco *et al.*, 1993) was used. The ability of adoptive transduced cells to infiltrate tumor tissues was examined. Athymic nude mice with N87 carcinoma xenografts were administered with MOLT-sFv23e-PE40 cells by tail-veil injection. 48 hr later, the mice were sacrificed and cryosections of tumor tissues were prepared and stained with anti-PEA antibody followed by an anti-goat IgG conjugate (Sigma). Significant fluorescent staining was observed in peripheral blood vessel regions of the tumor tissues of mice injected with MOLT-sFv23e-PE40, but not in animals injected with Molt-control cells. No apparent cytotoxicity to normal tissues (liver, lung, kidney, and heart) was observed under microscopy. The effects on tumor growth and animal survival were then determined. Molt-sFv23e-PE40 or Molt-control cells (0.5 to 1.0×10^7) were intravenously injected into the mice with N87 tumor xenografts weekly for six weeks. The administration of the transduced cells strongly retarded the growth of the tumor xenografts (FIG. 4A). The mice injected with Molt-control all died within 70 days, but the mice injected with Molt-sFv23e-PE40 all survived over

the observed period (FIG. 4B). Thus, administration of the transfected cells significantly inhibited tumor growth and prolonged animal survival.

EXAMPLE 3

Expression of Recombinant Anti-HIV-1 Toxin Fusion Protein

To generate an HIV-1-specific cytotoxic cell, a neutralizing human monoclonal antibody (F105) against the CD4-binding site of HIV-1 gp120 expressed on the surface of HIV-1-infected cells (Sodroski *et al.*, 1986; Lifson *et al.*, 1986) was used (Marasco *et al.*, 1993; Thali *et al.*, 1991; Chen *et al.*, 1994). The gene (PE40) encoding domain II (for translocation across membrane bilayer) and domain III (for adenosine diphosphate (ADP)-ribosylation of EF-2) of PEA (Gray *et al.*, 1984; Allured *et al.*, 1986; Siegall *et al.*, 1989) was fused to the κ chain gene of F105 (Chen *et al.*, 1994). Subsequently, a bicistronic expression vector (pCMV-Fab105-PE40) which contains a Fd chain (V_H+C_{H1}), internal ribosome entry-site sequence (IRES), and κ-PE40 chimerical gene was constructed (FIG. 5).

Cytotoxic cells specific for HIV-1-infected cells were generated by transducing the antibody/toxin expression vector into $CD4^+$ Jurkat human T-lymphocytes, followed by G418 selection. The G418-resistant cells were picked and subcloned, and genomic polymerase-chain reaction (PCR™) was used to detect whether the Fab105-PE40 gene was incorporated into the cells. As shown in FIG. 6A, the CMV promoter-Fd, Fd-IRES, IRES-κ chain, and toxin Domain III DNA fragments were specifically amplified from the genomic DNA isolated from the transduced Jurkat. Both Fd and κ-PE40 proteins were immunoprecipitated from the culture medium of Jurkat-Fab105-PE40 either by the anti-human IgG or anti-PEA antibody, indicating that the two fragments of Fd and κ-PE40 chain were assembled together into Fab fragments. Thus, the viable transduced lymphocytes are able to produce and secrete the antibody-PE40 molecules into culture medium.

The antigen binding activity of the secreted Fab105-PE40 was examined by an enzyme-linked immunosorbent assay (ELISA). Positive binding activity to gp120 was detected in the culture medium of Jurkat-Fab105-PE40, while no significant binding activity was detected in

Jurkat-Control. By comparison with binding activity of a serial dilution of purified parental F105 antibody (Chen *et al.*, 1994), about 0.6 to 0.7 μg/ml of the Fab105-PE40 molecules were produced from Jurkat-Fab105-PE40 (24 hr/1×10^{6}cells/ml). The toxin enzymatic activity of Fab105-PE40 was examined using an ADP-ribosylation assay. A significant ADP-ribosylation activity in the culture medium of Jurkat-Fab105-PE40 was detected, while only a background level of ADP-ribosylation activity was found in the medium of control cells. When compared with the ADP-ribosylation activity of purified PEA, approximately 0.8 μg/ml of PEA protein was produced from Jurkat-Fab105-PE40 (24 hr/1×10^6 cells/ml).

Several biological features of Jurkat-Fab105-PE40 were examined. When cell proliferation rates and viability of Jurkat-Fab105-PE40 and Jurkat-controls were examined, there were negligible differences. In addition, the DNA synthesis rates of Jurkat-Fab105-PE40 and Jurkat-control were similar, as measured by ^{3}H-thymidine incorporation with or without PHA (0.8 μg/ml) stimulation. The protein synthesis rates of Jurkat-Fab105-PE40 and Jurkat-control were also nearly identical as measured by ^{3}H-leucine incorporation. Thus, the transduced Jurkat-Fab105-PE40 cells maintained their basic biological functions.

Selective Cytotoxicity to HIV-1-Infected Cells

Selective cytotoxicity to HIV-1-infection of the transduced Jurkat-Fab105-PE40 cells was evaluated using co-cultivation assays to examine cell-killing activity against HIV-1-infected cells, and effects on viral reverse transcriptase (RT) production. To examine the cell-killing activity of the cytotoxic cells, parental Jurkat T-lymphocytes were infected with an HIV-1 laboratory strain (IIIB) or two primary HIV-1 isolates (INME and TPO), respectively, and were then co-cultivated with Jurkat-Fab105-PE40 or Jurkat-control at several different ratios in 12-well Costar-Transwell filter culture plates. The viable HIV-1-infected cells in the top-chamber were counted, and the percentage of non-viable cells is shown in FIG. 7A. Significant killing of the cells infected with strain IIIB or primary HIV-1 isolates was observed in the co-cultures with Jurkat-Fab105-PE40, but not with Jurkat-control. No adverse effects on un-infected parental Jurkat cells were observed.

To further observe the virus infection in the co-cultures, parental Jurkat cells were infected with an HIV-1 patient primary isolate (WEAU) for 6 days, washed with PBS three times, and then co-cultivated with Jurkat-Fab105-PE40 or Jurkat-control at several different ratios. HIV-1 infection in the co-culture was monitored every three to four days by measuring viral RT activity. As shown in FIG. 7B, in the co-culture with Jurkat-Fab105-PE40, only low levels of RT were observed throughout the period, while significantly higher levels of RT were detected in the co-culture with Jurkat-control. When increasing ratios of Jurkat-Fab105-PE40 to targeted HIV-1-infected cells were used, a stronger inhibition of HIV-1 infection was observed. Jurkat-Fab105-PE40 were not found to have any adverse effects on co-cultivated Jurkat and other lymphocyte lines not infected with HIV-1. Thus, Jurkat-Fab105-PE40 effectively inhibited HIV-infection, likely by the selective cytotoxicity and neutralizing activities of the Fab105-PE40 fusion proteins.

EXAMPLE 4

Generation of B-Cell Lineage-Specific Killer Cells

Recently a unique murine monoclonal antibody, Lym-1, which recognizes a polymorphic variant of the HLA-Dr antigen present on the cell surface of normal and malignant B-cells, was generated (Epstein *et al.*, 1987; Hu *et al.*, 1995). This antibody, Lym-1, is remarkably B-cell specific, with significantly increased avidity for lymphoma cells, when compared to normal B lymphocytes. Radioimaging studies with ^{123}I-labeled Lym-1 have demonstrated selective localization to the sites of lymphomas, as well as an ability to detect tumor sites (Epstein *et al.*, 1985; 1987; DeNardo *et al.*, 1987; 1988a; 1988b; Hu *et al.*, 1989). There has been no evidence of the Lym-1 antibody binding to T-cells or other normal cells and tissues as demonstrated by immunochemical detection of tissue sections and radioimaging (Epstein *et al.*, 1987). In summary, the antibody Lym-1 has been shown to be specific to malignant B-cells and can be used for targeted therapy for B-lineage malignancy.

In order to take advantage of the specificity of the Lym-1 antibody, killer cells specific for B-lineage leukemia/lymphomas were generated by transduction with a chimerical gene. The transduced cells recognize and kill B-lineage leukemia/lymphoma cells. Specifically, the

antibody variable region genes of the Lym-1 antibody were cloned and assembled into a single-chain antibody (sFv) gene. The sFv-Lym gene was fused in-frame to the domain II-III sequence of PEA, and cloned into a mammalian expression vector.

The specific cytotoxic T-lymphocytes, which are able to produce and secrete the sFv-Lym/toxin fusion protein, were generated by transducing the sFv-Lym/toxin gene with a leader signal peptide into human T-lymphocytes. The B-cell binding and toxin catalytic activities of the secreted antibody/toxin molecules were demonstrated by ADP-ribosylation assay and flow cytometric analysis. The selective cytotoxicity of the transduced cytotoxic cells to the B-cell leukemia/lymphoma cells were observed *in vitro*. The results demonstrate that this new class of cytotoxic cells with defined specificity may have broad applications for the treatment of B-lineage malignancy and other cancers.

Construction and Expression of Antibody/Toxin Fusion Proteins

The cDNA genes of the monoclonal antibody Lym-1 were obtained from Dr. A. Epstein at Univ. Calif. San Diego (Epstein *et al.*, 1987). The V_H and V_L cDNA genes were PCR™-amplified, respectively, and then gel-purified. An extension PCR™ was then used to assemble the V_H and V_L into a sFv. The resultant sFv-Lym protein consists of the light chain and heavy chain variable regions of the antibody Lym-1 linked by a polypeptide with a leader signal peptide sequence. The gene of PEA obtained from ATCC contains three functional domains: Domain I, cell-recognition; Domain II, translocation domain (amino acid (aa) residues 253 - 404); and Domain III, catalytic domain (aa 405 - 613) (Pastan, 1992).

Specifically, the variable region genes of the antibody Lym-1 were used as templates for PCR™ reactions as described previously (Chen *et al.*, 1994). A forward primer for V_H-Lym with an additional signal peptide leader sequence and a *HindIII* cloning site 5′-TTAAGCTTCATATGGAACATCTGTGGTTCTTCCTTCTCCTGGTGGCAGCTCCCAGA TGGGTCCTGTCC-3′ SEQ ID NO:22 (F-1)), and a reverse primer with an additional interchain linker sequence 5′-GCTCCCACCACCTCCGGAGCCACCGCCACCTGCAGAGACGTGACCCAGAGT-3′, SEQ ID NO:23, were used to amplify the V_H gene with a leader signal peptide sequence. A forward primer for V_L-Lym with an additional interchain linker sequence

5'-GGTGGCGGTGGCTCCGGAGGTGGTGGGAGCGGTGGCGGCGGATCTGAGCTTCGTG AATGACCCAGTCTCCA-3' SEQ ID NO:24, and a reverse primer with an additional *NotI* cloning site 5'-AAAGCGGCCGCACGTTTGATCTCCAGCTTGGT-3' SEQ ID NO:25, (F-2) were used to amplify the V_H gene with a leader signal peptide sequence. The PCR™-amplified V_H and V_L DNA fragments were then assembled into the sFv-Lym by extension PCR™ using the primers F-1 and F-2. The sFv-Lym with the leader sequence fragments were then cloned into the *HindIII/NotI* sites of a plasmid pRc/CMV (Invitrogen), and the resultant vector were designated as pCMV-sFv-Lym.

Generation of Cytotoxic Cells Specific for B-Lineage Leukemia/Lymphomas

Cytotoxic cells specific for B-lineage cells were generated by transduction of the fused antibody/toxin expression vector. The methods for gene transfer and selection were described previously (Chen *et al.*, 1994, 1995). Briefly, human T-lymphocytes such as Molt, or SupT, were transfected with the antibody/toxin expression DNA by electroporation, and selected in G418-contained culture medium. The G418-resistant cells were selected after two to three weeks, and subcloned. To determine the recombinant protein expression, the transduced cells were radiolabelled and immunoprecipitated as described previously. The samples were then analyzed by SDS-PAGE, and visualized by a PhosphoImager.

PCR™ analysis of genomic DNA from transduced lymphocytes was used to determine whether the antibody/toxin gene was incorporated into the genome of the transduced lymphocytes. The genomic DNAs were extracted from the transduced lymphocytes according to a standard method (Maniatias *et al.*, 1986). The oligonucleotides used for the PCR™ reactions, corresponding to the sFv-Lym and PEA toxin gene, are listed below: Pair A:; primer F-1 and 5'-TTTAAGATCTACAGGAGACGGTGACCGTGG-3' (SEQ ID NO:26); Pair B: F-2 and 5'-TTGCGGCCGCGAAAGGCGGCAGCCTGGCCGCG-3' (SEQ ID NO:27); Pair C: 5'-GGTACCGAATTCTCTAGAGGCGACGTCAGCTTCAGC-3' (SEQ ID NO:28), and 5'-TTAATTGCGGCCGCTTACTTCAGGTCCTCGCG-3' (SEQ ID NO:29). The PCR™ products were analyzed by agarose gel electrophoresis. Lymphocyte genomes that have incorporated the sFv-Lym/toxin encoding DNA are expected to generate three size specific bands with the PCR™ primer pairs as follows: an sFv-Lym DNA fragment of about 770 bp, a PEA domain II of about 450 bp and a PEA domain III of about 610 bp.

To examine the antibody/toxin expression in mammalian cells, a transient expression assay was performed. COS-1 cells were grown on coverslips, and 5 μg of the antibody/toxin expression plasmid DNAs were transfected into the cells by using lipofectin (Chen and Compans, 1992). After a 48 hour incubation, the cells were fixed and stained with an anti-toxin antibody (Gibco-BRL), followed by a fluorescent conjugate. A strong positive fluorescent staining was observed in the transfected cells, while no significant staining was found in the control cells. The fluorescent staining is located throughout the cytoplasm and perinuclear Golgi region, representing a typical secretory protein staining pattern. It is also evident that the transfected cells expressing the antibody/toxin proteins maintain their normal morphology, suggesting that mammalian cells are able to produce the antibody/toxin molecules, while remaining viable.

B Cell-Binding and Toxin Catalytic Activities of Secreted Recombinant Antibody/Toxin Fusion Proteins

To determine whether the secreted antibody/toxin fusion proteins maintain the antigen binding activity. Flow cytometric analysis were performed in malignant B-cell lines, such as Raji. Raji cells (1×10^6) (ATCC) were incubated with different serial dilutions of the antibody/toxin fusion proteins at 4°C for 30 min, and then incubated with an anti-mouse IgG antibody conjugate or an anti-PEA antibody followed by a labeled antibody conjugate (Sigma) for a period of time and under conditions that favor the development of further immunecomplex formation (e.g., incubation for 2 hours at room temperature in a PBS-containing solution such as PBS-Tween). T-cell lines, such as Molt-4, SupT, were used as a negative control. A positive result indicates that the antibody/toxin fusion protein produced by the lymphocytes is able to bind to B-cells.

EXAMPLE 5

Production of Targeted Toxins by Brain Cells

Plasmid DNA expressing TGF-PE40 fusion toxins are transfected into normal neurons via liposome encapsulation and the TGF-PE40 fusion toxins are produced and secreted from the

neurons. In a tumor xenograft nude mouse model, the transfected neurons are found to express the TGF-PE40 fusion proteins after one month of transfection.

The TGF-PE40 plasmid DNAs are also transfected into brain tumor cells in tissue culture and in tumor xenograft nude mouse models. It is found that the transfected tumor cells express the TGF-PE40, which results in the death of surrounding tumor cells with an amplified cytotoxicity, *i.e.*, a transfected cell is able to kill many surrounding tumor cells. Thus, targeted toxins may be used locally to produce and amplify a selective cell-killing ability, which may have broad implications for the treatment of cancer and autoimmune diseases, and particularly for the treatment of brain cancer. For example, after a tumor is removed from the brain, the plasmid/liposomes would be administered into the brain of the subject. The immunotoxins produced would then be able to target and destroy tumor cells left behind by the surgery that would not otherwise be detected until they had grown into another tumor. The present invention provides, therefore, a powerful therapy for the treatment of brain tumors.

EXAMPLE 6

Generation of Species Specific Killer Cells T-Helper Cells Associated with Autoimmune Diseases or Reactions

To transduce T lymphocytes so that they produce immunotoxins which are able to recognize antigens specifically associated with a subset of T-helper cells associated with an autoimmune disease or reaction such as arthritis, a blood sample, of approximately 200 cc/sample, is isolated from the subject. Lymphocytes are isolated from the blood sample and reared under appropriate conditions following standard protocols as exemplified by Janda et al., Manual of Clinical Microbiology, 5th Edition, American Society for Microbiology, Washington, DC, Chapter 19, p 137; (incorporated herein by reference). In this manner approximately 10^{11} lymphocytes may be isolated from culture after approximately two weeks.

Isolated cultured lymphocytes are transduced as described above such that they will produce and secrete a cytotoxin immunoreactive with the desired species of T helper cells. These lymphocytes may then be reinfused, or injected, back into the host subject in a

pharmaceutically acceptable carrier such that a dose of about 10^9 lymphocytes is delivered. The dosage may be readministered at intervals ranging from 2 weeks to 6 months or as desired. The site chosen for administration may be varied as appropriate to the condition being treated. For example, in the case of the inflammatory arthritis of a joint, it may be desirable to administer the dosage to the joint. Subcutaneous, intramuscular, intradermal or other sites may be used as deemed appropriate.

* * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: TARGETED CYTOTOXIC CELLS

(iii) NUMBER OF SEQUENCES: 29

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/740,003
(B) FILING DATE: 23-OCT-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
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(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
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(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(2) INFORMATION FOR SEQ ID NO: 23:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

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(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

GGTGGCGGTG GCTCCGGAGG TGGTGGGAGC GGTGGCGGCG GATCTGAGCT TCGTGAATGA 60

CCCAGTCTCC A 71

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

AAAGCGGCCG CACGTTTGAT CTCCAGCTTG GT 32

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

TTTAAGATCT ACAGGAGACG GTGACCGTGG 30

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

TTGCGGCCGC GAAAGGCGGC AGCCTGGCCG CG 32

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GGTACCGAAT TCTCTAGAGG CGACGTCAGC TTCAGC 36

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

TTAATTGCGG CCGCTTACTT CAGGTCCTCG CG 32

CLAIMS:

1. A mammalian cell that expresses and secretes an immunotoxin.

2. A mammalian cell of claim 1, further defined as a cytotoxic lymphocyte.

3. A mammalian cell of claim 1, further defined as a neuron.

4. A mammalian cell of claim 1, wherein said immunotoxin comprises an anti-tumor antibody domain.

5. A mammalian cell of claim 3, wherein said anti-tumor antibody domain is immunoreactive with a HER2 antigen.

6. A mammalian cell of claim 3, wherein said antibody domain is immunoreactive with a Lym-1 antigen.

7. A mammalian cell of claim 1, wherein the antibody domain of said immunotoxin is immunoreactive with a viral antigen.

8. A mammalian cell of claim 7, wherein said viral antigen is an HIV antigen.

9. A cytotoxic-T-lymphocyte of claim 2, wherein the antibody domain of said immunotoxin is immunoreactive with a T-helper cell.

10. A mammalian cell of claim 1, wherein the toxin of said immunotoxin is a one or more domains of a *Pseudomonas* exotoxin, diptheria toxin, ricin A, abrin, gelonin or saporin toxin.

11. A mammalian cell of claim 1, wherein said immunotoxin comprises a *Pseudomonas* exotoxin domain.

12. A mammalian cell of claim 1, dispersed in a pharmaceutically acceptable carrier solution.

13. A mammalian cell transfected with an expression vector, wherein said vector expresses a fusion protein comprising a leader sequence and an immunotoxin, and wherein said leader directs said immunotoxin into the endoplasmic reticulum of said cell.

14. A cell of claim 13, wherein said vector is a plasmid.

15. A cell of claim 13, wherein said vector is a viral vector.

16. A cell of claim 13, wherein said immunotoxin immunoreacts with a tumor associated antigen.

17. A cell of claim 13, wherein said immunotoxin immunoreacts with a viral associated antigen.

18. A cell of claim 13, wherein said immunotoxin immunoreacts with a T-helper cell associated antigen.

19. A cell of claim 17, wherein said viral associated antigen is an HIV associated antigen.

20. A cell of claim 19, wherein said HIV associated antigen is a gp120 antigen.

21. A cell of claim 16, wherein said tumor associated antigen is a HER2 antigen.

22. A cell of claim 16, wherein said tumor associated antigen is a Lym-1 antigen.

23. A cell of claim 13, further defined as a cytotoxic lymphocyte.

24. A cell of claim 13, further defined as a neuron.

25. A pharmacological composition comprising a cell of claim 13 dispersed in a pharmaceutically acceptable carrier.

26. A method of killing a cancer cell comprising contacting said cancer cell with an immunotoxin secreted from a mammalian cell, wherein the antibody portion of said immunotoxin recognizes an antigen of said cancer cell.

27. A method of claim 26, wherein said mammalian cell is a cytotoxic lymphocyte.

28. A method of claim 26, wherein said cancer cell overexpresses HER-2.

29. A method of claim 26, wherein said mammalian cell is a neuron.

30. A method of claim 26, wherein said cancer cell overexpresses Lym-1.

31. A method of claim 28, wherein said cancer cell is a breast, ovarian or gastric cancer cell.

32. A method of claim 26, wherein said cancer cell is a brain cancer cell.

33. A method of claim 26, wherein said cancer cell is in an animal subject and said mammalian cell is administered to said subject in a pharmaceutical composition.

34. A method of claim 33, wherein said subject is a human cancer patient.

35. A method of killing a virally infected cell comprising contacting said cell with an immunotoxin expressed from a cytotoxic-T-cell, wherein the antibody portion of said immunotoxin recognizes an antigen expressed by said virally infected cell.

36. A method of claim 35, wherein said cytotoxic-T-cell is administered in a pharmaceutical solution to an animal subject having a virally infected cell.

37. A method of claim 36, wherein said subject is a human.

38. A method of inhibiting an HIV infection in a subject comprising administering a pharmaceutical solution to said subject, wherein said solution comprises a cytotoxic-T-cell that expresses and secretes an anti-HIV immunotoxin.

39. A method of inhibiting tumor cell growth in a subject comprising administering a cytotoxic-T-cell expressing an anti-tumor cell immunotoxin to said subject.

40. A method of producing a recombinant immunotoxin comprising the steps of:

obtaining a mammalian cell transfected with an expression vector, wherein the vector expresses a fusion protein comprising a leader sequence and an immunotoxin, and wherein the leader directs the immunotoxin into the endoplasmic reticulum of the cell; and

culturing said cell under conditions effective to express said immunotoxin.

41. The method of claim 40, further comprising the step of isolating said immunotoxin.

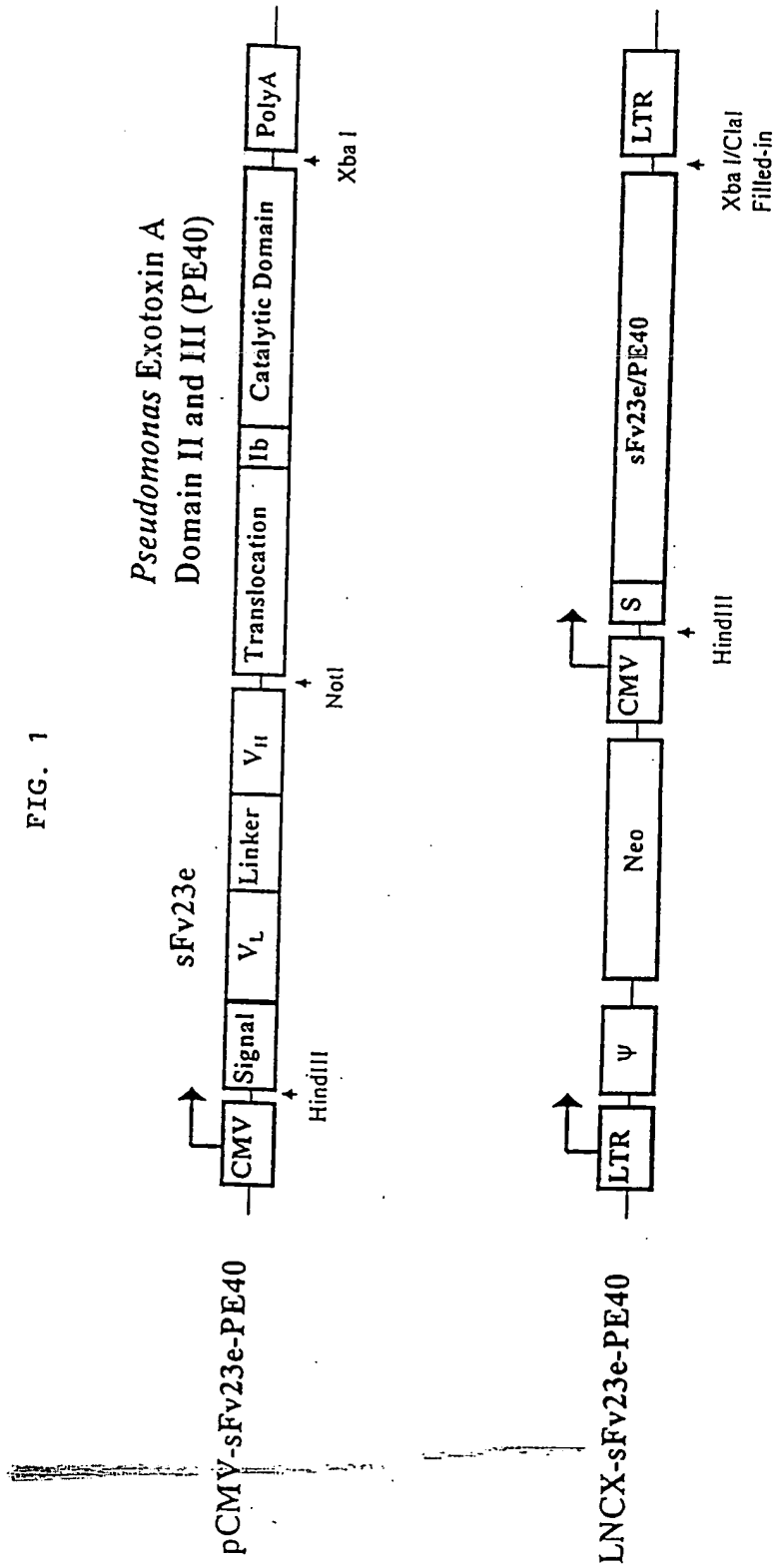

Pseudomonas Exotoxin A
Domain II and III (PE40)
sFv23e
CMV
Signal
V_L
Linker
V_H
Translocation
Ib
Catalytic Domain
PolyA
HindIII
NotI
Xba I
pCMV-sFv23e-PE40
LTR
ψ
Neo
CMV
S
sFv23e/PE40
LTR
HindIII
Xba I/ClaI
Filled-in
LNCX-sFv23e-PE40

FIG. 1

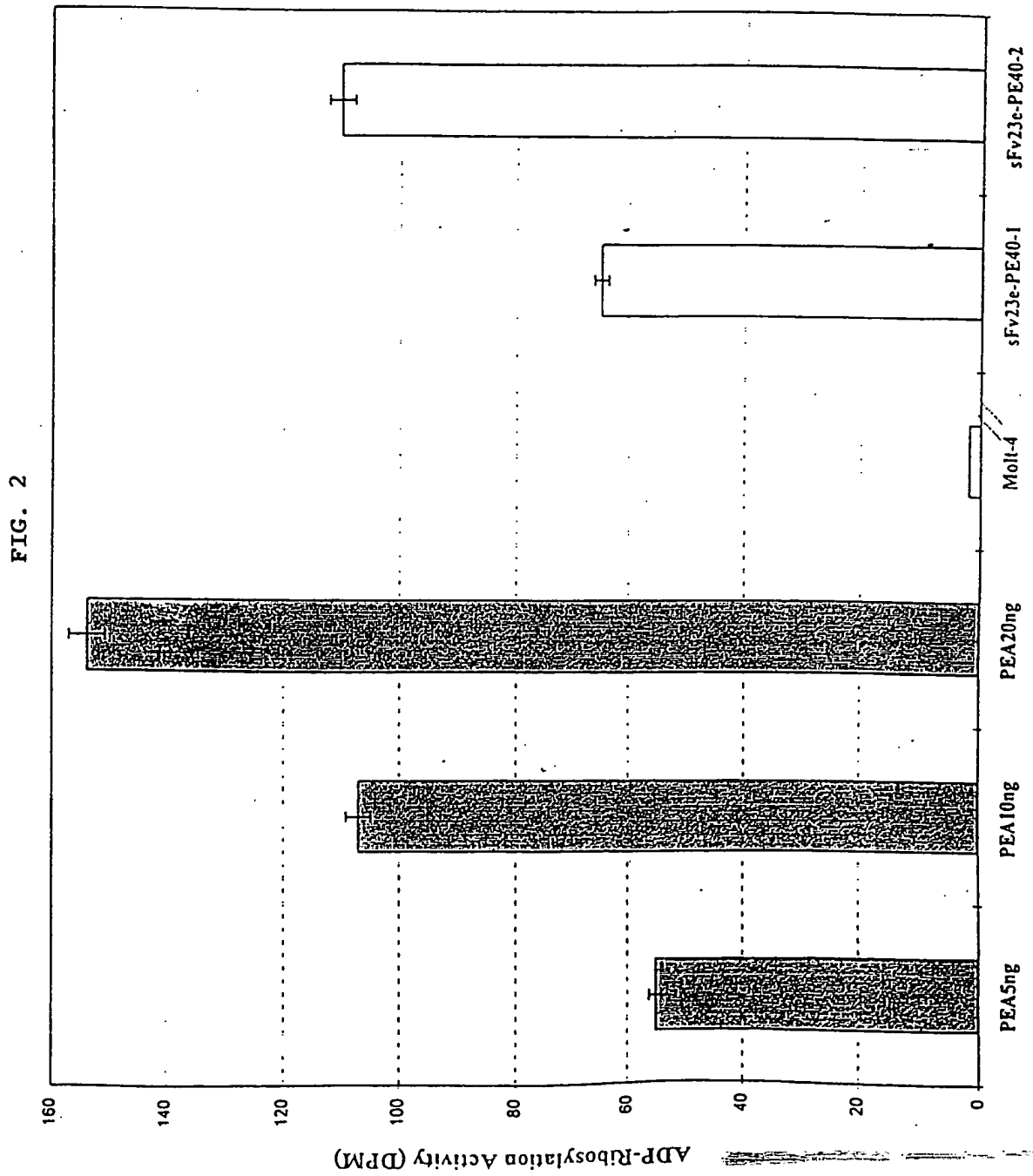

ADP-Ribosylation Activity (DPM)
0
20
40
60
80
100
120
140
160
PEA5ng
PEA10ng
PEA20ng
Molt-4
sFv23e-PE40-1
sFv23e-PE40-2

FIG. 2

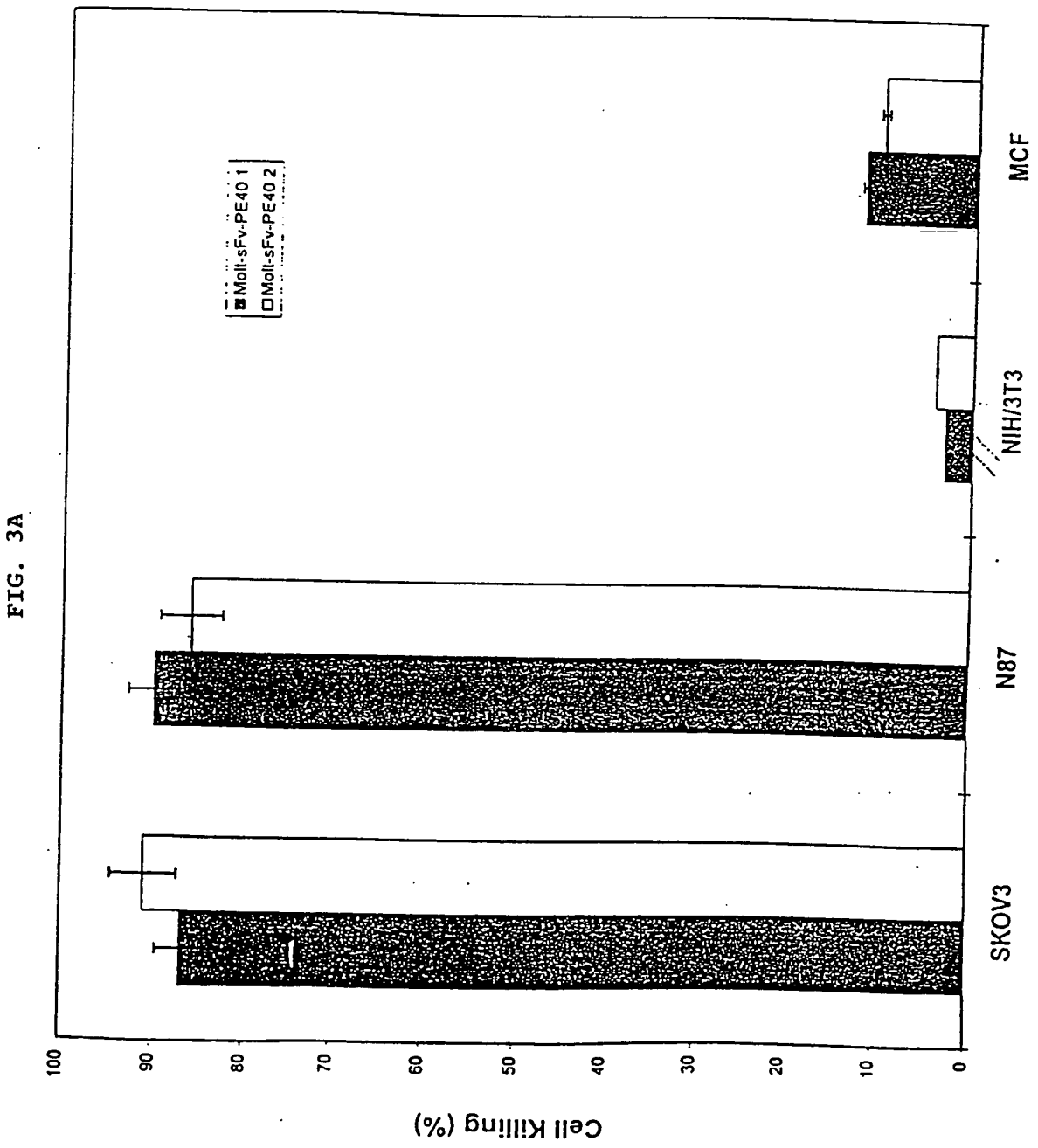

Cell Killing (%)
100
90
80
70
60
50
40
30
20
10
0
SKOV3
N87
NIH/3T3
MCF
Moll-sFv-PE40 1
Moll-sFv-PE40 2

FIG. 3A

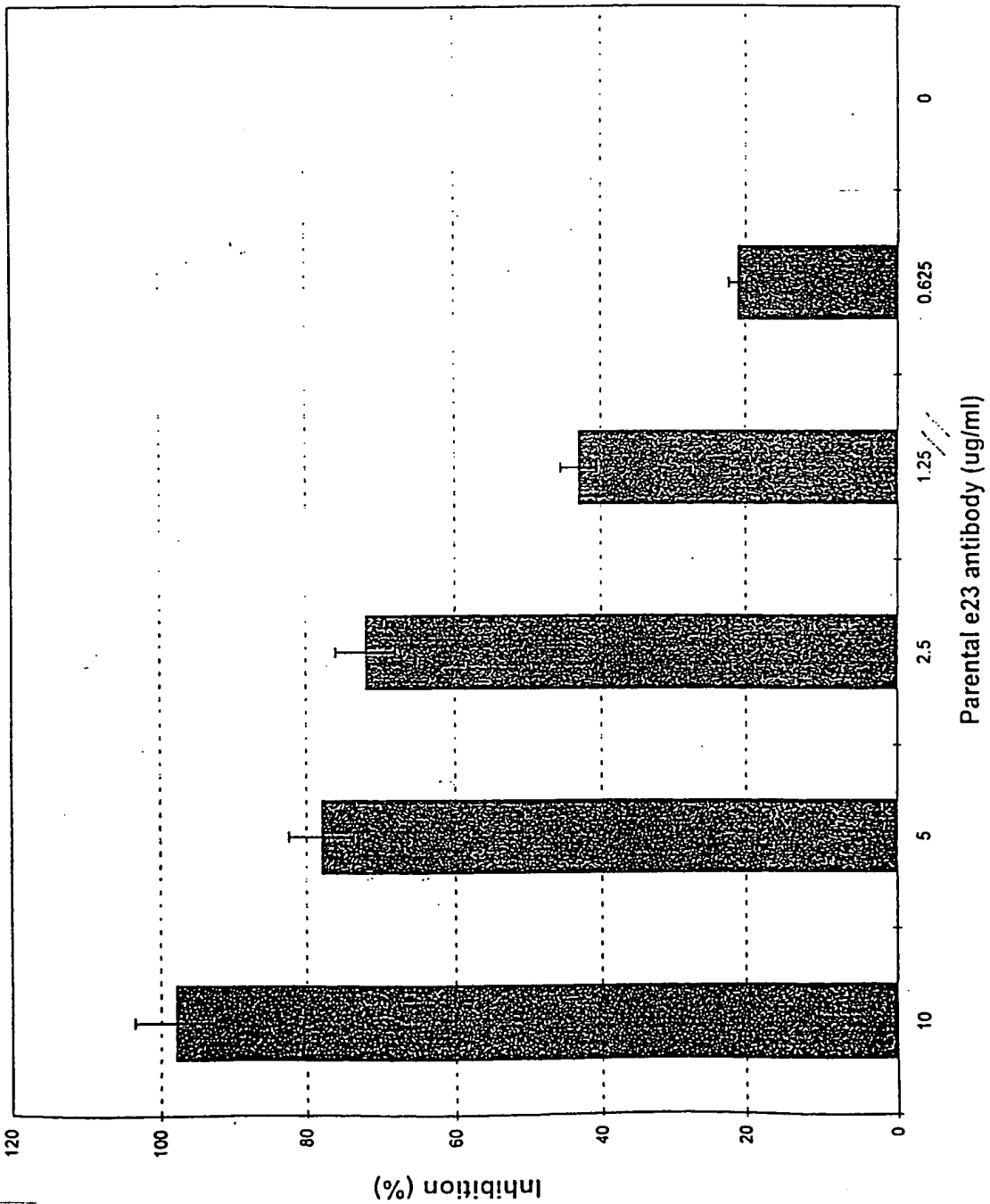

Inhibition (%)
120
100
80
60
40
20
0
Parental e23 antibody (ug/ml)
0
0.625
1.25
2.5
5
10

FIG. 3B

Transduced Cells
Control Cells
Immunotoxins
Control/PBS
Tumor Volumn (cm³)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
Days Posttreatment
1
3
5
7
9
11
13
15
17

FIG. 4A

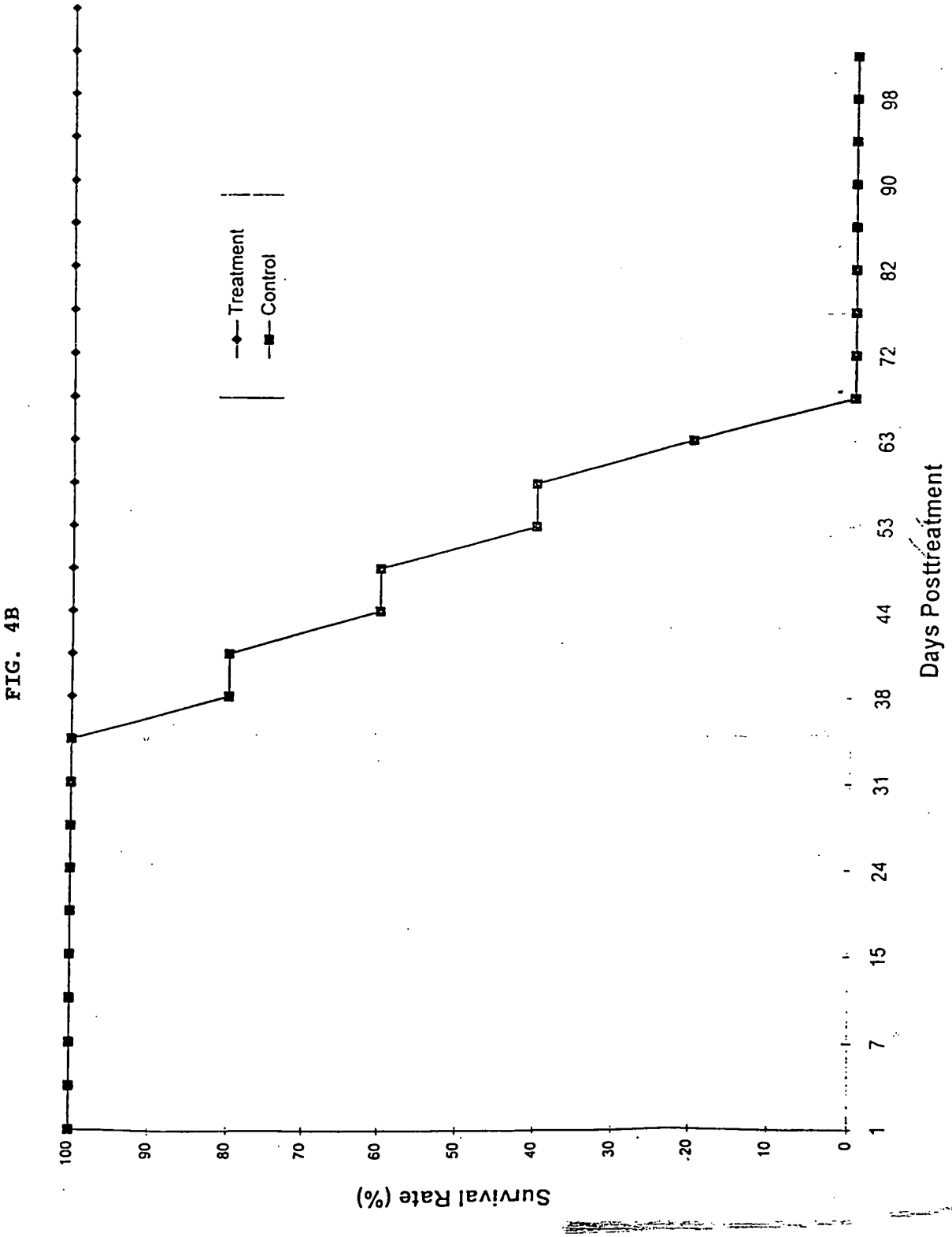
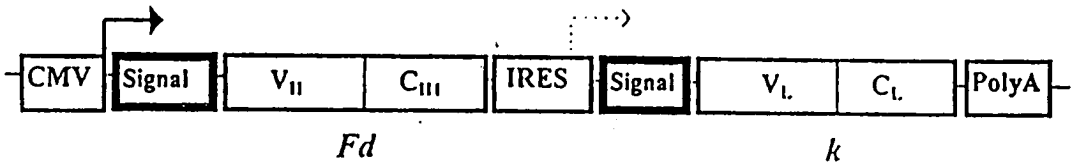

Survival Rate (%)
100
90
80
70
60
50
40
30
20
10
0
Days Posttreatment
1
7
15
24
31
38
44
53
63
72
82
90
98
Treatment
Control

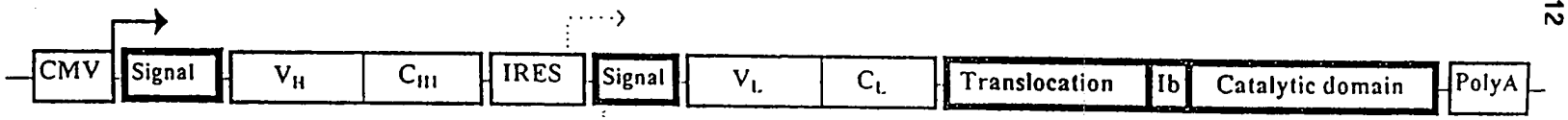
FIG. 4B

FIG. 5

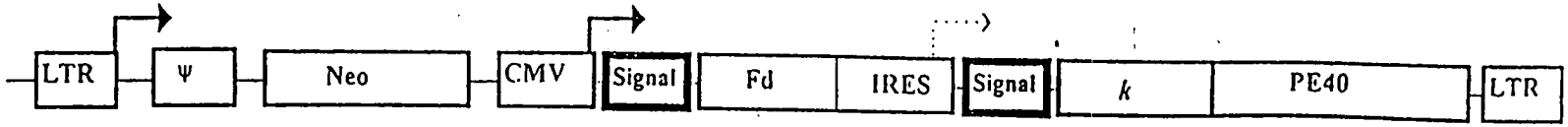
pCMV-Fab105I



pCMV-Fab105-PE40



LNCX-Fab105-PE40



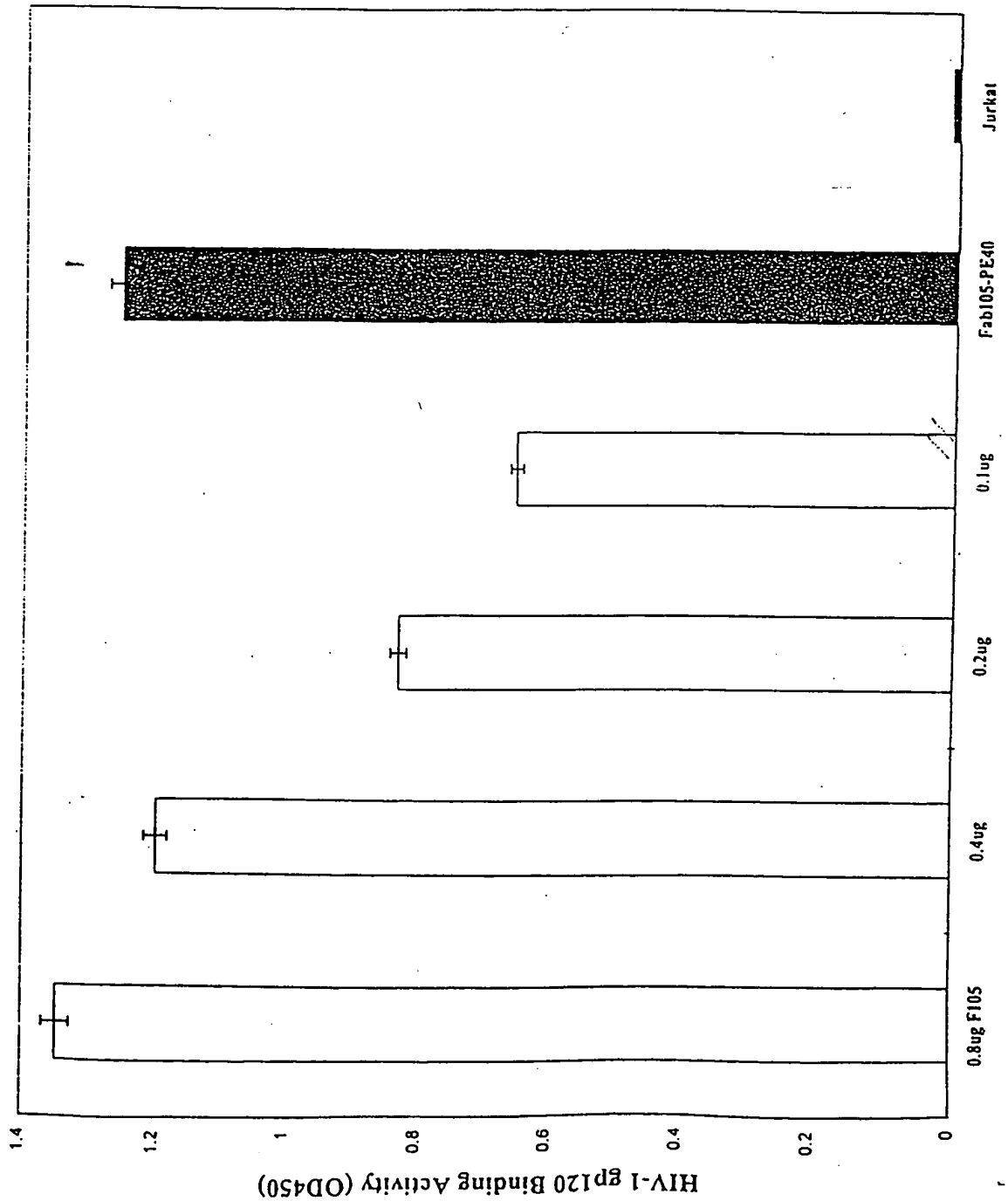

HIV-1 gp120 Binding Activity (OD450)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.8ug F105
0.4ug
0.2ug
0.1ug
Fab105-PE40
Jurkat

FIG. 6A

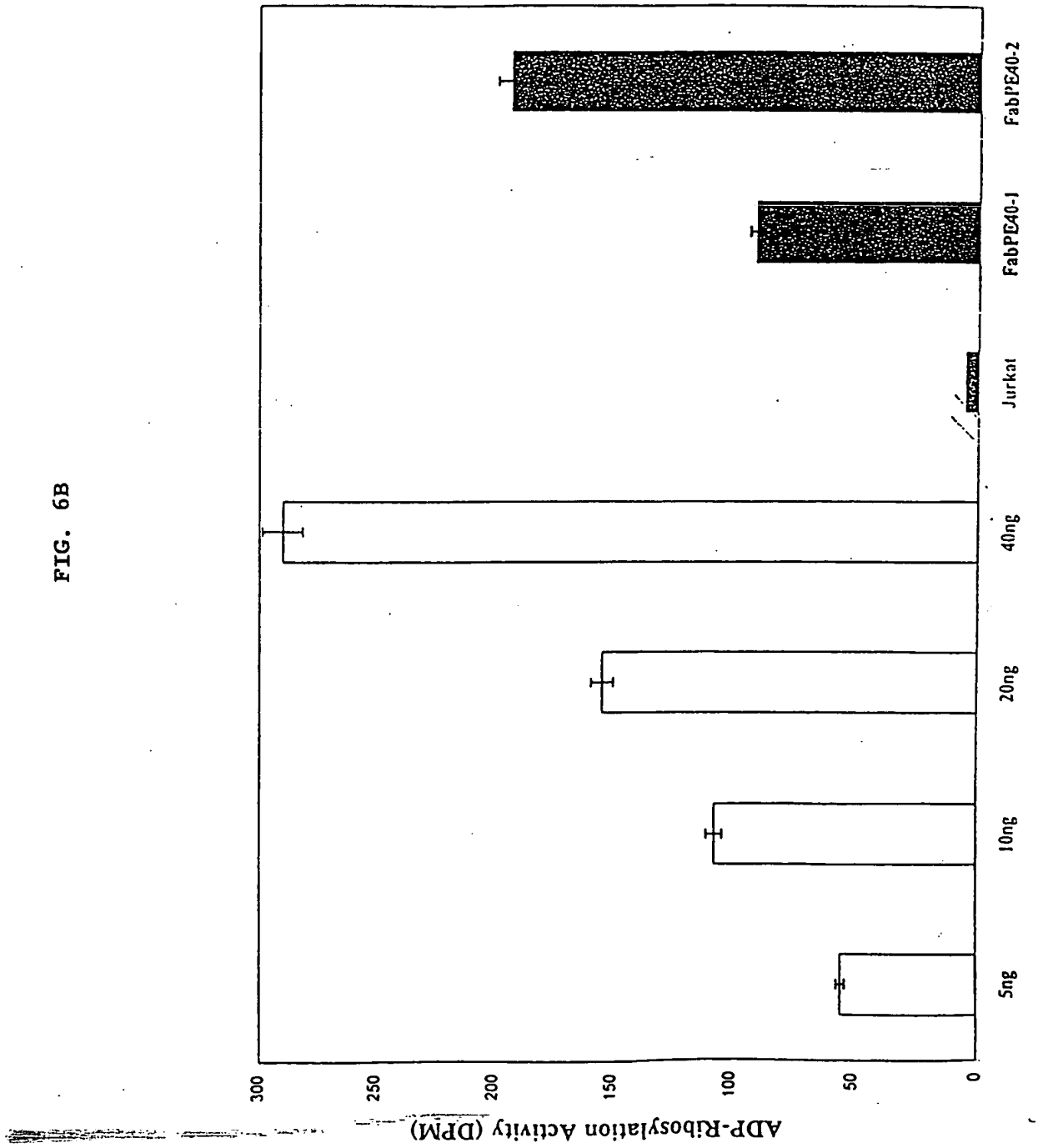

ADP-Ribosylation Activity (DPM)
300
250
200
150
100
50
0
5ng
10ng
20ng
40ng
Jurkat
FabPE40-1
FabPE40-2

FIG. 6B

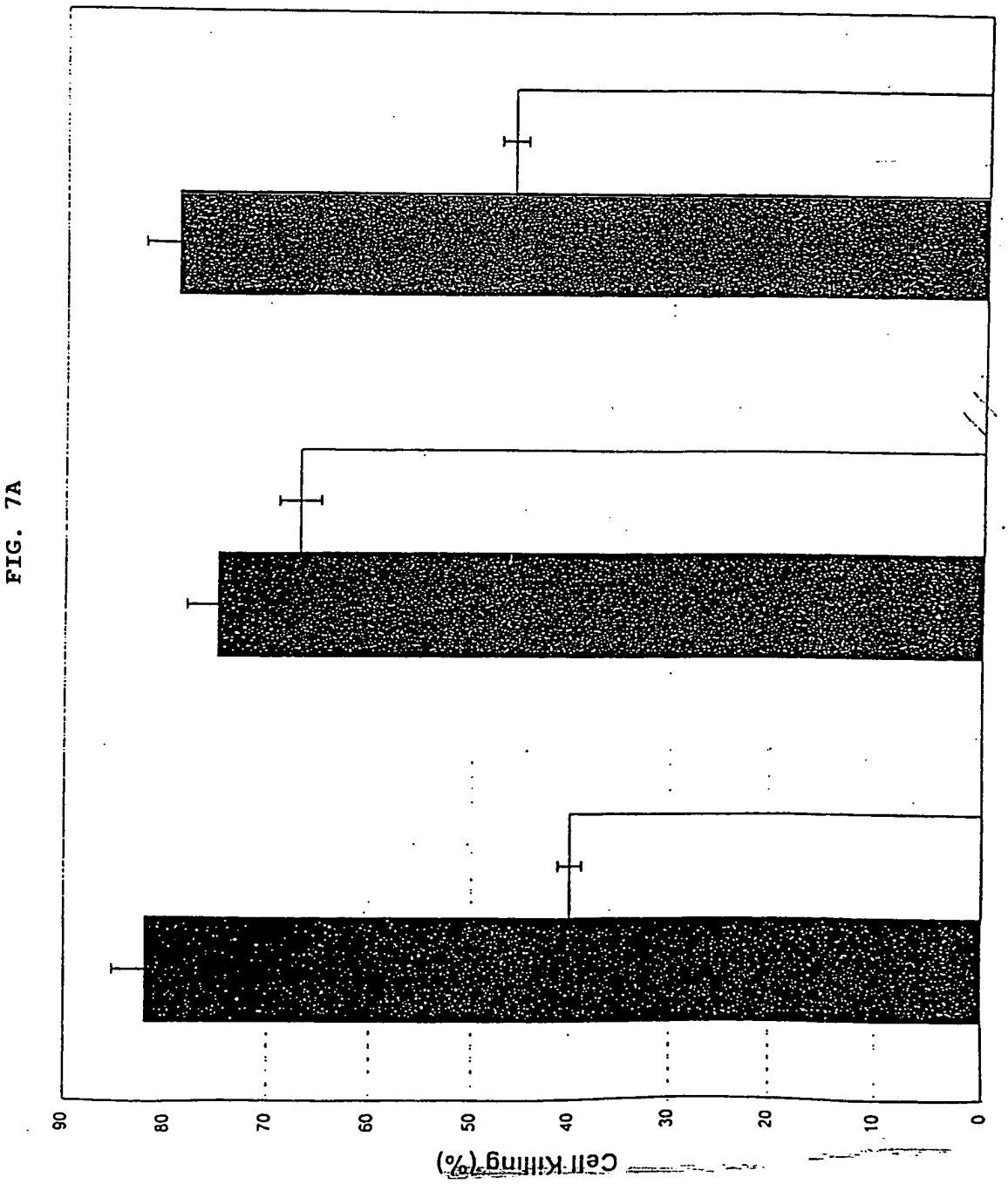


FIG. 7A

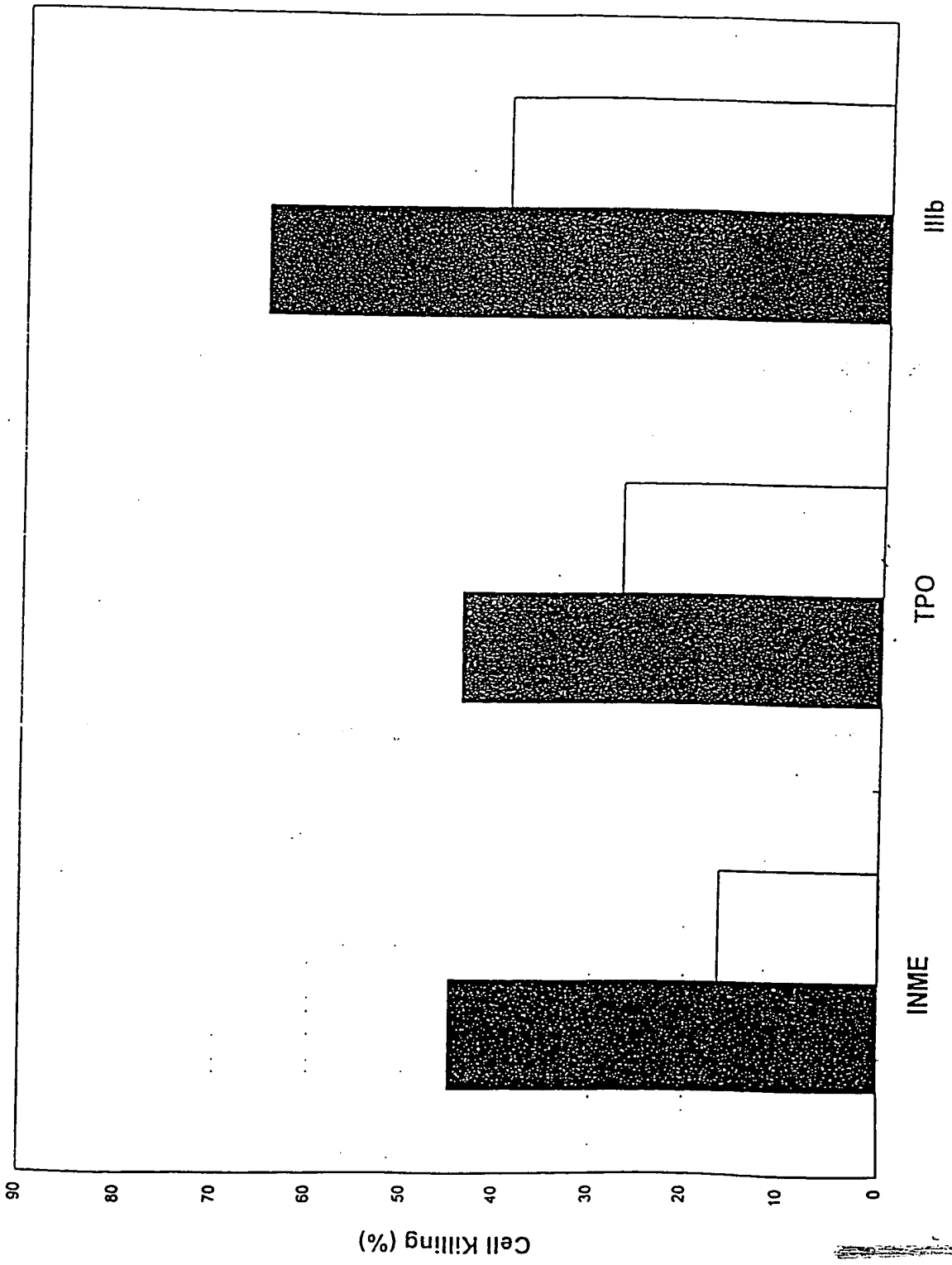


FIG. 7B

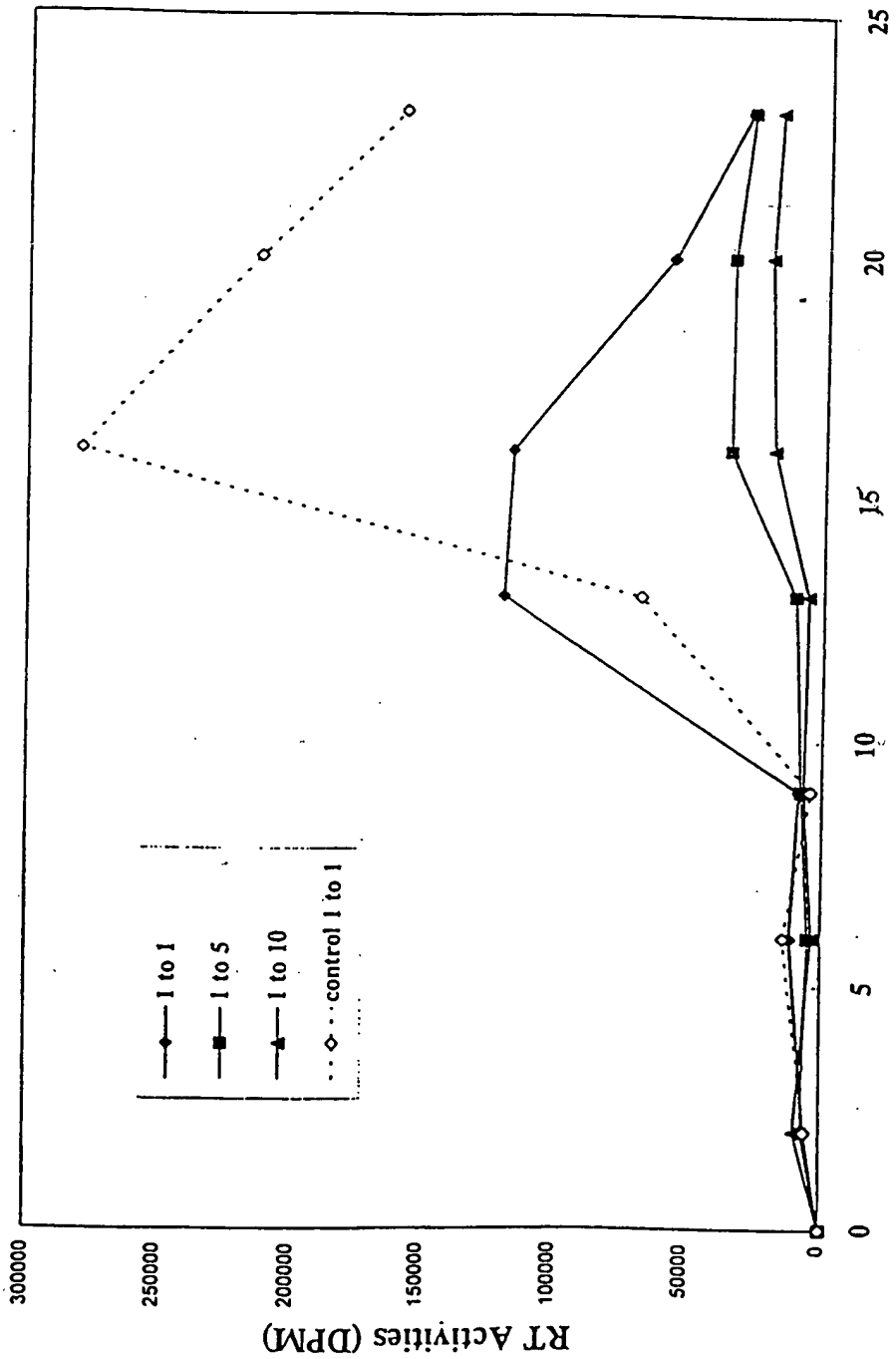

RT Activities (DPM)
300000
250000
200000
150000
100000
50000
0
0
5
10
15
20
25
Days After Co-cultivation
1 to 1
1 to 5
1 to 10
control 1 to 1

FIG. 7C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19206

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.2, 93.21, 93.7, 93.71, 178.1, 183.1; 435/69.1, 69.7, 325, 326, 328, 352, 363, 365, 365.1; 530/391.1, 391.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	WO 90/12592 A1 (THE UNITED STATES OF AMERICA, as represented by, THE SECRETARY U.S. DEPARTMENT OF COMMERCE) 01 November 1990, see entire document.	1, 4, 7, 10, 11, 13, 14, 16, 17, 26-27, 31, 32, 39-41 ---------- 2, 3, 5, 6, 8, 9, 12, 15, 18-25, 28-30, 32-38

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
07 JANUARY 1998

Date of mailing of the international search report
29 JAN 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
RON SCHWADRON
Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19206

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEN et al. Combined intra- and extracellular immunization against human immunodeficiency virus type 1 infection with a human anti-gp120 antibody. Proceedings National Academy of Sciences USA. June 1994, Vol. 91, pages 5932-5936, see entire document.	1, 2, 4-23, 25-41
Y	CAI et al. Genetically marking human cells--Results of the first clinical gene transfer studies. Cancer Gene Therapy. 1995, Vol. 2, pages 125-136, see entire document.	1-39

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/19206

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A01N 63/00; A61K 38/00, 38/16, 39/00, 39/395, 48/00; C07K 14/00, 16/46, 19/00; C12N 5/00, 5/08, 5/06, 5/10, 15/00, 15/85, 15/86

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/93.2, 93.21, 93.7, 93.71, 178.1, 183.1; 435/69.1, 69.7, 325, 326, 328, 352, 363, 365, 365.1; 530/391.1, 391.7

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, BIOSIS, EMBASE, DERWENT WPI, APS, CHEM AB, search terms:author names, killer cell, LAK, CTL, TIL, T cell neuron, CHO, COS, mammal, cell, toxin, immunotoxin, conjugate, immunoconjugate, transfected, transduced, HER 2, tumor, HIV, secreted, expressed